A STUDY ON THE CONTROL OF INTERFERON PRODUCTION IN LYMPHOBLASTOID CELLS.

One Volume.

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SUMMARY.

This research was aimed at increasing the yield of lymphoblastoid interferon produced by the Namalwa line of lymphoblastoid cells. The parameters involved in the induction and production of interferon were studied first to give basic biochemical information about interferon and viral production in Namalwa cells. Optimised conditions were used for the remaining studies. The Namalwa cell line was cloned from single cells to determine whether there was any variation in interferon production in a population of cells. No significant variation was found so a study was made with cells treated with 5'-bromodeoxyuridine and butyric acid. These chemicals alter gene activity in many other cell types. The study was made using the mechanism of superinduction as a basic model for the control factors involved in interferon production. Neither chemical showed identical biochemical effects to the anti-metabolites used to effect superinduction in fibroblast cells. Thus it appears that the two chemicals act in a different manner to anti-metabolites. Neither compound affected the replication of the inducing virus. Further investigation showed that 5'-bromodeoxyuridine required incorporation into cellular DNA before increased interferon yields were obtained. The effect of butyric acid on interferon yields could not be directly associated with any of its varied biochemical and morphological effects.

These results have increased out knowledge of lymphoblastoid cells and indicated that interferon production in these cells may be controlled in a similar, though not identical way, to the model proposed for fibroblast interferon from fibroblasts. Two chemicals have been shown to increase the yield of interferon from Namalwa cells but the biochemical basis of their activity has not been resolved.

ABBREVIATIONS.

ATP	Adenosine Triphosphate.
5'-BrdUrd	5' - bromo - deoxyuridine.
BT	Bovine Turbinate.
CEF	Chick embroyo fibroblasts.
cDNA	complementary DNA.
Ci	Curie.
CldUrd	5' - chloro - deoxyuridine.
cAMP	cyclic adenosine monophosphate.
cpm	counts per minute.
DABA	(3',5 - Diaminobenzoic acid dihydrochloride).
DcAMP	dibutyryl - cyclic adenosine monophosphate.
DNA	deoxyribonucleic acid.
DNase	5,6 dichloro - 1 -β - D - ribofuranosyl -benzimadazole.
dATP	deoxy - adenosyl - triphosphate.
dCTP	deoxy - cytidyl - triphosphate.
dGTP	deoxy - guanosyl - triphosphate.
dTTP	deoxy - thymidine - triphosphate.
DMSO	dimethyl sulphoxide.
EBV	Epstein-Barr Virus.
EBNA	Epstein-Barr Nuclear Antigen.
EDTA	Ethylene diamine tetra acetic acid.
eIF-2	eukaryotic initiation factor 2.
FCS	foetal calf serum.
FS-4	foreskin fibroblasts.
FSH	follicle stimulating hormone.
FdUrd	5' - fluorodeoxyuridine.
G6PdH	Glucose 6 phosphate dehydrogenase.
GMEM	Glasgow modified Minimal Essential Medium.
HCG	Human choronic gonadotrophin.
HFF	Human foreskin fibroblast.
HVJ	Haemagglutinating Virus of Japan.
HA/HAU	Haemagglutinin (units).
IdUrd	5' - iodo - deoxyuridine.
HLeIF	Human Leukocyte Interferon.
HFIF	Human Fibroblast Interferon.

$\log_{10}$	logarithm to base 10.
MDBK	Malin-Darby bovine kidney.
MELC	Murine erythroleukaemia.
MLV	Murine leukaemia virus.
MMTV	mouse mammary tumour virus.
mRNA	messenger RNA.
MSV	Murine sarcoma virus.
$NaHCO_3$	sodium bicarbonate.
NaOH	sodium hydroxide.
NCS	new born calf serum.
NDV	Newcastle disease virus.
NIH	National Institute of Health Bethesda Maryland U.S.A.
PBS	phosphate buffered saline.
pH	$-\log_{10}$ hydrogen ion concentration.
pI	isoelectric focussing point.
PCA	perchloric acid.
pfu	plaque forming units.
poly(rA)	polyribo-adenylic acid.
poly(rU)	polyribo-uridylic acid.
poly(rI).poly(rC)	polyribo-ionsinic acid and polyribo-cytidylic acid (double stranded RNA).
POPOP	1,4-di(2(5-phenyl-oxazolyl))-benzene.
PPO	2,5-Diphenyloxazole.
RNA	ribonucleic acid.
RPMI	Roswell Park Memorial Institute.
SFV	Semliki Forest Virus.
SDS	sodium dodecyl sulphate.
SV5	Simian Virus type 5.
SV40	Simian Virus type 40.
TAT	tyrosine aminotransferase.
TCA	trichloro-acetic acid.
TEMED	N,N,N^1,N^1 - tetramethylenediamine.
UV-NDV	ultraviolet irradiated Newcastle disease virus.
VSV	vesicular stomatitus virus.
v/v	volume per volume.
wt/vol	weight per volume.

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DECLARATIONS.

Work published from this thesis is contained in the paper bound in at the end of this thesis. The title of the paper is "The effect of 5'-Bromodeoxyuridine on the interferon production by Human cells". This work is presented in Chapter 3. My work in the paper consisted of all the work referring to Namalwa cells. The main conclusions of the joint work is presented in Chapter 3 for comparison only.

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INTERFERON - Its Biological properties.

Interferon was discovered by virtue of its ability to inhibit viral growth in tissue culture (Isaacs and Lindenmann 1957). Interferon is active in a wide variety of cells, both *in vitro* and *in vivo*, but appears to be species specific - thus human interferon is active on human cells but not on mouse or chick cells. The inhibition of viral growth in tissue culture cells required pre-treatment of the cells with interferon before infection with virus for its maximum effect. Cells treated with interferon are resistant to infection by a wide variety of viruses and the cells are said to be in an 'antiviral state'. Few viruses are resistant to the actions of interferon.

Apart from the antiviral activity, interferon also exhibits effects on cellular functions (Dahl 1977). Firstly interferon can enhance or depress its own production in certain cells. These actions are termed 'priming' and 'blocking' respectively (Stewart, Gresser and Lockhart 1971a, 1971b). Secondly interferon slows cell growth. This is referred to as the 'anticellular effect' (Stewart *et al* 1976). Cells vary in their sensitivity, and tumour cells are also sensitive to the 'anticellular effect'. Cells such as L1210 mouse leukaemia cells are inhibited in their growth *in vitro*, in their ability to form colonies in agar and in their ability to form tumours *in vivo* (Gresser *et al* 1970, 1971). The anticellular effect has been observed with the most highly purified preparations of interferon and appears to be a real property of the molecule. Interferon has also been shown to enhance the toxicity of the double-stranded RNA, poly(rI).poly(rC), for cells (Stewart *et al* 1972).

Interferon has at least two clinically useful properties - an antiviral effect and an anti-tumour effect. Only human interferon will be of any potential use in man.

The potential clinical use of interferon.

Tissue culture studies show that interferon holds promise as a wide spectrum antiviral agent. Such an agent has long been sought

after and could be compared to the wide spectrum antibiotics, like penicillin, which have had a big impact on disease. Furthermore, interferon has antitumour activity which may, or may not, be a result of its antiviral activity. Interferon is, however, in limited supply. The immediate targets for the clinical use of interferon are those infections that cannot be treated at present, such as viruses with many non cross-reactive serotypes (e.g. rhinoviruses), latent infections such as herpes simplex, where recurrent infections often occur, and chronic infections such as hepatitis B. The immediate effect of interferon should be to protect non-infected tissues and so prevent the spread of the virus. Thus in viral infections interferon must be given either early in infection or prophylactically to be of any use. It is anticipated that subsequent to the suppression of infection by interferon other normal body defences may overcome the infecting agent and effect a complete cure. At the present time it is impossible to treat prophylactically with interferon because of the scarcity of the material.

Limitations on clinical use.

Interferon is produced in vivo in response to certain infections. In order to reach the levels of interferon seen in tissue in vivo, for the purpose of clinical administration, it is necessary to use large and frequent doses of exogenous interferon. Interferon is rapidly cleared from the bloodstream after injection, though its fate is not known and it is inactivated by many body fluids. The doses normally given are more than 10^6 units of interferon per patient, per day, for the systemic infections. The activity of interferon appears to be in the region of 10^8 - 10^9 units per milligramme and thus this kind of dose is equivalent to about 10 microgrammes (see Pollard and Merigan 1979).

The usage of interferon is restricted by the small amounts of interferon that are made by cell cultures (up to about 10^5 units/millilitre) and the very small proportion that interferon represents of the total protein in the preparations. This means that relatively large scale handling is required and the subsequent purification of

interferon is difficult. The majority of the interferon used clinically is made from leukocytes isolated from whole blood (Cantell 1978). About 10^{11} units of interferon can be made per year. Thus relatively few patients can be treated at present, particularly where long term trials are required. This interferom has been successfully used to treat osteosarcoma patients in Sweden (Strander et al 1973). The purity of these preparations has recently reached about 5×10^{6} per milligramme or about 1% (Cantell and Hirvonen 1978). However, there do not appear to be any severe side reactions caused by the impurities to date (Pollard and Merigan 1979).

The immediate possibilities for increasing production of interferon must lie with much larger scale handling, continuous culture production, the use of high producing cells, or indeed a combination of these factors.

In the long term interferon provides a prime target for genetic manipulation. The subsequent production of interferon in bacteria could achieve higher production, both by virtue of the higher cell densities that can be achieved and the conversion of a large proportion of cellular synthetic activity to interferon production. The latter can be achieved by the insertion of many copies of the interferon gene into the cell.

The antiviral activity of interferon.

Two approaches that could potentially increase the availability of interferon for clinical use can be conceived. Either the amount of interferon per culture of cells could be increased, as indicated above, or the antiviral activity of interferon could be increased.

Increases in the effective antiviral concentration of interferon can be produced either by altering the protein itself, or by increasing the specificity of the interaction of interferon with the target tissue.

The interferon receptor.

It is known that interferon must be externalised from the producing cell before it is active (Vengris, Stollar and Pitha 1975). This could simply result from its compartmentalisation within the cell (see later). However, there is an interaction at the cell surface which involves a cellular receptor of some kind (Chany et al 1975, Slate and Ruddle 1978). Presumably some cells are not sensitive to interferon because they do not possess these receptors. An increase in the number of receptors per cell may increase the sensitivity of the cells to the effects of interferon. Receptors of differing affinities have been proposed to account for the differing interactions of different species of interferon on the same kind of cell (Edy, Billiau and DeSomer 1976, Gardener and Vilcek 1979). Since the structure of interferon may soon be known (Weissenbach et al 1979) a knowledge of the structure of these receptors might allow the synthesis of more specific "interferons" by chemical modifications of purified interferon to give a more specific interaction. It is assumed that the interferon-receptor interaction is similar to the hormone-receptor systems that have been well characterised. Polypeptide hormones such as insulin use a 'secondary messenger' as an amplification system (Butcher, Robinson and Sutherland 1972). It is not known if interferon requires a second messenger or how many molecules of interferon are required to induce the antiviral state. Both insulin and interferon (Gresser 1977) are known to have many effects on the cell surface including alterations of transport of certain molecules into the cell.

Liposomes offer a potentially useful delivery system for both interferon itself (La Bonnardiere 1979) or for the interferon inducers such as poly(rI).poly(rC) (Magee et al 1976). As yet they lack the tissue specificity that would be required to give very localised high concentrations of interferon but they do offer protection to the messenger molecules from inactivation.

The antiviral mechanism.

The mode of inhibition of viral replication by interferon is not well understood. There is evidence that viral replication can be inhibited at three sites - transcription, translation and maturation. The first observation of an inhibition of primary transcription was vesicular stomatitis virus (VSV) (Marcus et al 1971). The most convincing studies in this area are those done on simian virus type 40 (Oxman and Levin 1971, Metz, Levin and Oxman 1976). It appears that the inhibition of primary transcription is not enough to account for the marked inhibition of viral replication.

Inhibition of viral translation has been observed in intact cells and in cell-free systems. For VSV and reovirus it has been shown that only a slight transcription inhibition occurs so that an inhibition of translation may occur (Yamamoto, Yamaguchi and Oda 1975, Wiebe and Joklik 1975). The cell may discriminate between viral messenger RNA (mRNA) whose translation is inhibited, and cellular mRNA, which is not inhibited, by making modified cellular mRNA's that are larger than normal (Levy and Riley 1973). In cell-free systems a low molecular weight inhibitor of translation has recently been identified as a novel oligonucleotide (Kerr and Brown 1978, Ball and White 1978). This oligonucleotide has also been shown to activate an endonuclease (Clemens and Williams 1978). The selective phosphorylation of two proteins occurs in the presence of ATP. They have molecular weights of 67,000 and 35,000. The latter may be one of the eukaryotic initiation factors eIF-2, which is required for initiation of protein synthesis (Revel 1977). The activation of all these components requires interferon pre-treatment of intact cells and the addition of double-stranded RNA to the cell-free system made from the cells. Double-stranded RNA is thus suggested to cause the activation of three 'enzyme presursors' which are converted to a specific kinase, an endonuclease and the oligonucleotide synthetase (Baglioni 1979). However, it appears that the latter enzyme is made de novo and requires transcription before it is observed in interferon treated cells (Ball 1979),

while neither the activation nor the activity of the endonuclease requires protein synthesis (Ratner et al 1977, Vaquero and Clemens 1979). Furthermore, a new protein of 56,000 molecular weight is observed. While the oligoadenylate synthetase activity copurifies with this protein, the two have not been shown to be identical (Ball 1979). (It is interesting to note that this is the first clear evidence that gene activity is controlled by interferon treatment.) The specificity of the resulting inhibition of translation by these factors remain to be shown.

This system is not restricted to the interferon induced antiviral state. The production of the three molecules found in interferon treated cell extracts also occurs in crude rabbit reticulocyte lysates in the absence of interferon treatment, but in response to double-stranded RNA (Farrell et al 1977, Vaquero and Clemens 1979). They are also found in L cells (Clemens and Williams 1978). Addition of purified eIF-2 in the reticulocyte system does not overcome the inhibition of translation, whereas the addition of new mRNA does. This suggests that the endonuclease activity is the important factor in the inhibition of the translation (Vaquero and Clemens 1979). The oligoadenylate varies with the hormone (oestrogen) status of the tissue (Stark et al 1979). Thus the oligoadenylate and its associated endonuclease may represent a mechanism for degrading cellular mRNA's in preparation for assuming specialised functions and particularly these molecules may regulate tissue regression and differentiation (Hovanessian and Kerr 1978, Stark et al 1979).

Recent evidence suggests that interferon can also inhibit certain viruses at the stage of maturation and release. RNA tumour viruses such as murine leukaemia virus (Billiau et al 1974, 1975; Friedman and Ramseur 1974, Friedman et al 1975 and Pitha et al 1976), mouse mammary tumour virus (Wong et al 1977 and Chang et al 1977) and SV5 (Billiau et al 1978) appear to be inhibited at this stage. Such an inhibition is not restricted to RNA tumour viruses since VSV particles with low infectivity are made in interferon treated cells and there is a preferential inhibition of the release of infectious particles (Maheshwari and Friedman 1979). The particles only contain full genomes and are not thus defective. Interferon alters the virion

structural component(s) or changes the properties of the cell plasma membrane. Changes in the cell membrane have been observed after interferon treatment (Lindahl et al 1973, Vengris et al 1976 and Chang et al 1978).

It thus appears that the interferon mediated inhibition of viral replication does not have a single mechanism but several, possibly to cope with the wide variety of viruses.

The antiviral mechanism seems to use cellular regulatory molecules that are not interferon specific. Thus, at present, there does not appear to be any specifically modifiable system that would allow an increase in the antiviral activity to be realised. The mechanism of the induction of the antiviral state is not known so neither can this stage be controlled. Thus the best possibility of realising the potential clinical use of interferon is to make higher specific activity preparations.

To this end I shall consider what is known about the regulation of interferon production. It is not possible to exhaustively review all the data here, and there are many recent reviews of this kind in any case. Therefore I shall confine myself to the key ideas that offer the potential for increasing the yields of interferon from cells.

Firstly, then, let me consider the nature of interferon since an understanding of this suggests the type of control by which the molecule is likely to be regulated.

The nature of the interferon molecule.

Interferon is a protein and is probably glycosylated (Weil and Dorner 1973). The molecular weight of interferon is not precisely known but appears to be in the region of 20,000. Determination of the molecular weight is difficult because glycoproteins behave aberrantly on polyacrylamide gels and because there are several biologically active molecular sizes, that behave as interferons, in interferon preparations. This latter effect is called 'microheterogeneity' and may arise as a result of three possibilities. Either interferon is bound to a range of other proteins, or it has differing amounts of carbohydrate residues

per molecule, or there is more than one protein sequence that possesses antiviral activity. There is evidence that each of these possibilites occurs and accounts for the 'microheterogeneity' observed for interferon.

Purification of interferon from crude preparations removes the contaminating proteins and reduces, to some extent, the microheterogeneity. Interferon is now known to bind to a range of proteins such as bovine serum albumin with a high affinity and such binding properties have been exploited as a method for purifying interferon (Huang et al 1974).

Interferon is also present in forms that differ in their amount of glycosylation. Preparations can be treated in vitro with glycosidases. This treatment reduces the number of species of interferon detectable (Bose et al 1976). Periodate can also be used to specifically remove carbohydrate residues, although this agent can attack amino acids and the resulting activity of the interferon preparation is low (Stewart et al 1977). In the intact cell inhibitors of glycosylation have been used (Havell et al 1975, Mizrahi et al 1978 and Fujisawa, Ikwakura and Kawade 1978). All these treatments show that some of the microheterogeneity of interferon preparations seem to be due to differing extents of glycosylation. Although it cannot be proven that a few carbohydrate residues remain on the protein after these treatments, it seems that the non-glycosylated interferon retains activity. Thus the carbohydrate residues do not seem to be required for biological activity. The biological significance of glycosylation is not yet known for any protein (Kornfeld and Kornfeld 1976). It has, however, been suggested that glycosylation particularly the presence of sialic acid residues, may protect the protein against degradation (Dorner, Scriba and Weill 1973). The fact that interferon is a glycoprotein may account for the fact that it penetrates poorly into tissues after injection.

Not all the microheterogeneity can be removed using the methods above, and there is evidence to suggest that interferon activity is associated with more than one protein primary sequence. Interferons from different species can be distinguished antigenically, so the

primary sequence does not appear to be highly conserved as a whole. Human interferons can also be sub-classed antigenically. There appear to be three types of human interferon by this criterion. At least two of these types are made in different cells. Fibroblasts make human fibroblast interferon (HFIF) and leukocytes make human leukocyte interferon (HLeIF). These interferons can be used to protect the same kind of cells but one type is generally more active than the other on a given cell type (Edy et al 1976 and Gardener and Vilcek 1979).

Human interferon also demonstrates some deviation from what was considered a general property of interferons - that of species specificity. HLeIF is active in bovine and feline cells, sometimes more active than on human cells, whereas HFIF is not active in these cell types (Gresser et al 1974, Desmyter and Stewart 1976 and Edy et al 1977). This is not a property confined to human interferon but it does appear that interferons have a restricted host range in terms of activity (Stewart 1979).

Direct evidence that there are at least two structural genes for interferon comes from studies on an human lymphoblastoid cell line. These cells produce interferon that is mostly HLeIF, but a small proportion of the antiviral activity is not precipitated by anti-HLeIF antibody but is precipitated by anti-HFIF antibody and thus appears to be HFIF (Pancker 1977 and Havell, Yip and Vilcek 1978). Both types of interferon are also made if interferon messenger RNA is extracted from these cells and translated in Xenopus oocytes. The interferon made in the oocytes shows the same proportion of the two types of interferon as made by the intact lymphoblastoid cells. It is unlikely that the oocyte has two different glycosylation systems for a single protein and therefore this result suggests that the two interferons are structurally different (Cavalieri et al 1977). Thus some of the heterogeneity of interferon preparations may not result solely from differences in glycosylation.

A third type of human interferon is known that has all the properties of an interferon (see Lockhart 1966) except that it is unstable at pH 2 and produced in response to different inducers (see later). This interferon is antigenically different from the

other two human interferons which are stable at pH 2 (Valle et al 1975).. The pH 2 stable species are referred to as Type I interferons and the pH 2 unstable interferon as Type II. Type II interferons are also found in other species and can be identified both in vitro and in vivo. It is not known which lymphoid cell type produces Type II interferon. The existence of human Type II interferon implies, but does not prove, that there are at least three human structural genes for interferon. Another possibility is that splicing occurs within a single gene.

An important point to bear in mind when interpreting the data is that the antisera used are not raised against purified interferons. This is because there is insufficient pure interferon to use. Thus the antigen(s) involved in the precipitation reactions are not precisely known.

The genetics of the interferon system.

It has previously been indicated that there are probably at least three structural genes for human interferon. Extensive work has been undertaken both in vitro and in vivo. All the methods rely on the assumption that the yield of interferon is directly related to the genotype. As pointed out above, variations can occur that are not thus related.

Two approaches have been taken in vitro. The production of somatic cell hybrids by fusion is the major tool. These hybrids preferentially lose chromosomes from one of the parent cell types. Thus mouse-human hybrids retain only a few human chromosomes. This process is continual so there is a problem with the stability of the karyotype in these experiments. The first hybrids were made between nucleated chick erythrocytes and two human cell lines. Of the three parental cell lines only one of the human cell lines made interferon. When this interferon producer was fused with the chick cell the resultant hybrid made both human and chick interferons. The non-producing human line when fused made no interferon at all (Guggenheim, Friedman and Rabson 1968). Enhanced expression of interferon genes thus presents a possibility for making high

producers. This has been achieved with human-hamster (Morgan and Faik 1977) and mouse-hamster (Carver, Seto and Midgeon 1968, Smith and Sedak 1975). For the former set of hybrids human chromosome 18 controls the production of hamster interferon.

Chromosome assignments for human fibroblast interferon have suggested that two genes are required for production. These loci are situated on chromosomes 2 and 5 (Tan 1977). Interferon production in hamster-human hybrids provides evidence that the structural gene locus is on chromosome 5 and chromosome 2 contains a regulatory locus. Recent evidence conflicts with these data and suggests that chromosome 9 is solely required for the expression of interferon (Meager et al 1979).

Aneuploid cells have been used to study gene dosage effects i.e. by comparing cell lines having varying numbers of copies of certain chromosomes or partial deletions of chromosomes. The data show that the long-arm of chromosome 5 has the structural gene locus while the short-arm has a locus for the repressor or similar regulatory function (Tan 1977). High producing cell lines have been selected by cloning out cells containing multiple copies of chromosome 5, and these have been used to provide good material which can be purified to homogeneity (Berthold et al 1978).

Studies in vivo have been restricted to mice where two loci determining interferon production have been identified. Different loci were subsequently identified for different inducers (DeMaeyer et al 1974).

General consideration of the regulation of the interferon system.

Interferon is an inducible protein. Inducible proteins are specialised proteins whose synthesis is not normally required by the cell. The classic example of an inducible system is the β-galactosidase gene studied in E. coli. β-galactosidase is not normally made until the cell encounters lactose as a source of carbon and energy. Work on this system resulted in the operon model for regulation at the level of the DNA (Jacob and Monod 1961). Other examples which conform to this hypothesis are found in

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prokaryotes but evidence is lacking to support this hypothesis in eukaryotes. Many inducible systems are found in eukaryotes, some for one or a few proteins such as the interferon system and others giving rise to larger scale changes in biosynthetic activity within cells, such as those processes under hormonal control e.g. progesterone/oestrogen (O'Malley *et al* 1969) or those falling into the broad category of differentiation (Rutter *et al* 1973). In all these cases there is *de novo* synthesis of new mRNA's. Induction of protein essential to the cell also occurs. One example is the induction of tyrosine aminotransferase (TAT) activity above basal level by cortisone (Tomkins *et al* 1966). In this case there is no evidence that there is any increase in the levels of TAT mRNA.

The interferon inducer.

The inducer for interferon can be one of many substances. The most common inducers are viruses and the synthetic double-stranded RNA poly(rI).poly(rC). TypeII interferon can be stimulated by many diverse molecules including bacterial endotoxins and several low molecular weight molecules - for a comprehensive list of inducers see Kleinschmidt (1972).

Not all cells can be induced for interferon and furthermore not all potential interferon producing cells respond to all inducers. Particular cell lines do not give rise to the same yields of interferon when compared to other cell lines or the effect of different inducers. Yields can vary with differing preparations of a single inducer and with the culture conditions of the producing cells. The processes behind these variations are not known, though some of the possibilities have been put forward (see Stewart 1979, page 80).

If large scale production of interferon can be achieved then it is obviously important to control these variations. The unravelling of the processes that regulate interferon production would allow much better control of yields.

The role of the inducer.

The interaction of cells with the inducer is not understood. The steps involved in triggering interferon production are unknown. In the case of poly(rI).poly(rC) induction the site of induction has still to be resolved (Pitha and Pitha 1973). It is generally accepted that induction involves the derepression of the interferon gene(s). How this event is connected, if at all, with the various effects of double-stranded RNA - the postulated inducer - which occur concurrently with the induction is not known (Torrence and Friedman 1979).

Other phenomena associated with induction are priming and hyporeactivity. Priming occurs after pretreating cells with low doses of interferon resulting in higher titres after induction than normal. The mechanism underlying priming is not understood but it appears that the cells become more sensitive to double-stranded RNA (Kerr *et al* 1974). Priming does not occur if the interferon treatment is given at the same time or later than the inducer. Pretreatment with interferon is thus required. This also alters the membrane properties of cells so priming could possibly be associated with an increased uptake of inducer, which presumably has a membrane receptor.

Hyporeactivity is a diminished interferon response to an inducer which can occur very quickly after induction (Breinig, Armstrong and Ho 1975). Thus it seems unlikely that interferon itself is involved in this phenomena. However, in view of the persistence of the effect it also seems unlikely that the inducer *per se* is involved.

The production of interferon after induction.

Once induced the rate of interferon production reaches a maximum in a matter of hours and then declines again to undectectable levels. This corresponds to the situation described by the Jacob and Monod model but is unlike terminal differentiation, an example of which is the induction of haemoglobin synthesis in erythrocytes, where

haemoglobim synthesis is not switched off. The kinetics of interferon production varies with the cell type and the inducer (Stewart 1979). Priming seems to shorten the period after induction during which interferon is undetectable (Fleischman 1977).

Treatment of the producing cells with antimetabolites during induction and interferon production originally showed that interferon is made de novo and has a messenger RNA. The use of particular dual regimens, e.g. actinomycin D and cycloheximide (Tan et al 1970), gave paradoxical results - interferon yields were actually increased. This phenomena is called 'superinduction'.The kinetics of interferon production show an earlier detectable response to the inducer and a longer period of interferon production (Vilcek and Kohase 1977). Not all cells can be superinduced, though the reason is not known. Priming and superinduction are sometimes synergistic. Superinduction has only been observed when using poly(rI).poly(rC) or Ultra-violet irradiated Newcastle Disease virus. Ultra-violet irradiation of the cells before induction can also result in superinduction (Vilcek, Havell and Kohase 1976).

Recently it has been reported that other agents can alter the interferon yields from cells. Both 5'-bromodeoxy ridine - an analogue of the DNA precursor thymidine - (Tovey et al 1977) and sodium butyrate - a short chain fatty acid - (Johnston et al 1979) can increase yields of interferon, while retinoic acid - a vitamin A derivative - inhibits interferon production (Blalock and Gifford 1976).

The interferon system departs from the β-galactosidase system in that interferon cannot be produced continually in the presence of inducer. If further inducer is added after the initial production has occured then little or no further interferon is produced by the cell (Vilcek 1962 and Vilcek and Rada 1962). Cells do not recover from this hyporeactive state for a period usually of several days after interferon production has occured (Breinig, Armstrong and Ho 1975). The processes that result in hyporeactivity are not known.

Summary.

Interferon is a cellular glycoprotein which must be induced before synthesis takes place. Interferon is made by translation of a specific mRNA. The extent of translation is controlled by a shut off mechanism which prevents continuous production and further induction. However the use of certain agents can increase interferon yields.

The selection of high producing cell lines could be achieved in one or more of the following ways.

The selection of a highly efficacious inducer would endure that all cells were producing interferon and at their normal maximum rate. Studies on the nature of the inducer could lead to the selection or synthesis of more active or effective inducers.

Isolation and amplification of the structural gene(s) for interferon using genetic manipulations could provide increased yields of interferon per cell.

The rate of transcription of the interferon gene to give interferon mRNA could be increased. This could include a change in the rate of processing of a precursor RNA which represents the primary transcript.

The rate or period of translation might be increased. This could be achieved either by increasing the amount of functional interferon mRNA, as above, or by altering the efficiency of translation. The latter could result from an increase in the competition of interferon mRNA for ribosomal binding sites compared to other messenger RNA's or by increasing the rates of the initiation, elongation and termination of the polypeptide chain.

Changing the rate or extent of post translational modification or secretion may also permit higher extra cellular levels of interferon to be achieved.

I shall now consider what is known about these possible regulatory sites, as far as the interferon system is concerned, in more detail.

The regulatory sites in interferon production.

The nature of the inducer.

The identity of the inducing molecule has been studied extensively. At first sight the sheer diversity of the types of inducers does not present an obvious candidate for the inducing molecule. The inducers known to date have been variously classified. Grossberg (1977) has listed the following classes:

Type 1 inducers.

Viruses.
Double stranded RNA's - synthetic and animal cell derived.
Intracellular microbes.
Microbial products.
Chemicals - polymers and low molecular weight compounds.

Type 11 inducers.

Antigens - in sensitized immunocytes.
Mitogens - 'T' and 'B' lymphocytes.

For Type 1 inducers it has been suggested that double stranded RNA is the inducing component (Kleinschmidt 1972). Negative stranded viruses, such as Newcastle Disease virus (NDV), are potent inducers. Such single stranded RNA viruses form double stranded replicative intermediates synthesized by the virion associated RNA dependent RNA polymerase (Field et al 1967). Ultraviolet irradiated NDV(UV-NDV) is a more potent inducer than the wild type virus, although it is unable to replicate (Ho and Breinig 1965). UV-NDV still has RNA dependent RNA polymerase activity and produces base-paired RNA both in vitro and in vivo (Clavell and Bratt 1971). The synthetic double stranded RNA poly(r[illegible]).poly(rC) is a well known inducer and the structural requirements for induction have been exhaustively tested (Levy 1977). Reovirus, which contains double stranded RNA segments in the intact virion is also an inducer.

Vaccinia virus, which is a DNA containing virus, both induces interferon and forms a double-stranded RNA replicative intermediate. Of the non double-stranded RNA inducers some of the fungal inducers such as statolon have either associated mycophages containing double-stranded RNA, or have other associated RNA.

The case against double-stranded RNA has also been put. Double-stranded RNA from intact non interferon producing cells can be isolated and used to induce other cells (DeMaeyer et al 1971 and Kimball and Duesberg 1971). Other studies have shown either no correlation between the amount of double-stranded RNA produced by an inducer and its inducing ability (Lockart et al 1968, Thacore and Younger 1970 and Meager and Burke 1972) or even an inverse correlation (Lomniczi and Burke 1970). Vaccinia virus double-stranded RNA will not induce interferon in chick cells either (Bakay and Burke 1972). This has led some people to suggest that other viral functions are required for induction (Gauntt 1973, Lai and Joklik 1973, Atkins and Lancashire 1976 and Kowal and Younger 1978). Other non double-stranded RNA inducers do not give rise to interferon in whole animals - only in tissue culture cells. It has been suggested that these agents may act by perturbing the immune system, to which the interferon system is closely linked (Gresser 1977).

The most elegant demonstration of the role of double-stranded RNA in interferon inductions come from the use of defective mutants of VSV (Marcus and Sekellick 1977). One such mutant (±VSV) consists of a double-strand of complementary RNA representing both message and virion sense RNA covalently linked together to form a hairpim structure. In the absence of detectable transcription from this RNA it was found that a single molecule per cell of this ±VSV mutant was required for interferon induction.

Thus it seems that if a unifying hypothesis can be formulated, double-stranded RNA is the most likely candidate molecule to be cast in the role of the inducer.

Assays for the interferon messenger RNA.

Early work with metabolic inhibitors showed that both RNA synthesis (Heller 1963, Wagner and Huang 1965, Field et al 1967 and Seghal, Tamm and Vilcek 1975) and protein synthesis (Tan, Armstrong and Ho 1971) was required for interferon production and that interferon was itself sensitive to proteases. This suggested that interferon is a protein and has a messenger RNA.

The interferon system has few structural genes. Thus any measurement of transcription of the interferon gene(s) requires a very specific and sensitive assay. Potentially the most sensitive assay for messenger RNA (mRNA) results from the use of a highly labelled and specific probe. This probe can be made from the purified mRNA using the ensyme reverse transcriptase and labelled deoxynucleotides. A complementary DNA (cDNA) is thus synthesized which will specifically hybridise to any RNA carrying the mRNA sequences. This thus allows the primary transcription products to be assayed regardless of any post-transcriptional modification such as processing and degradation. For the interferon system it has not been possible to purify the interferon mRNA. Therefore the approach described above cannot be taken.

The assays used to date are: cell-free translation, heterologous cell systems and microinjection of Xenopus oocytes. Only interferon that is translated in these systems to give biologically active interferon can at present be identified. This RNA may not represent the primary transcript. Thus comparisons of interferon mRNA levels between different cell types is qualitative since they rely on the producing systems making recognisable interferon mRNA and have associated problems of nuclease activity, competition with other mRNA's both from the producing and assaying cells, and the possibility that differing post-translational modification and degradation will alter the final titre of interferon obtained.

The first system used to measure interferon mRNA was the heterologous cell system (DeMaeyer-Guignard et al 1972). Messenger RNA preparations can be added to tissue culture cells and these cells

produce active interferon that has the same species specificity and antigenic properties of interferom made in the producing cell from which the mRNA was isolated. Interferon characteristic of the assay cell is not produced. The mRNA is apparently absorbed by the cell. The ability of DEAE-dextran to increase the sensitivity of the assay (Kronenberg and Friedman 1975) is consistent with the idea that it increases the absorption of RNA by cells (Tovell and Colter 1967). It is likely that only a small proportion of the mRNA activity added to the cells is assayed and differential absorption is a possible problem with this assay from a quantitative point of view.

Cell-free translation systems have been used to obtain similar results (Pestka et al 1975, Raj and Pitha 1975 and Thang et al 1975), see also page 28 .

Xenopus oocytes have also been used (Reynolds, Premkumar and Pitha 1975, Cavalieri et al 1979 and Morser et al 1979). This system has certain advantages. Only about 1% of the RNA required for cell-free translation gives comparable synthesis and post translational modifications can be carried out in the oocyte. Post-translational modifications may be required to give biologically active interferon. However there are disadvantages. One is the microinjection technique required to put RNA into the cell. RNA may consequently not enter the cell or leak out after the injection. The method of selection is not known and neither is it known if all mRNA's are translated with equal efficiency. The titre of interferon produced thus depends on competition between interferon mRNA and other cellular RNA's that are coinjected and the oocyte's own mRNA's.

These assays have shown, invariably, that non-induced cells do not contain translatable interferon mRNA. It is not possible to completely rule out two possibilities - namely that interferon mRNA is present in an inactive form in uninduced cells with the inducer bringing about its activation, or that interferon mRNA is constituitively made but rapidly degraded in uninduced cells (Vilcek and Kohase 1977).

Superinduction - a post-transcriptional event ?

It is possible to alter the amount of interferon made in certain cell types. The interferon yield can be depressed or increased according to the treatment used. It is interesting to note that interferon itself can both increase and depress its own synthesis depending on the dose used for pretreating the cells (Stewart, Lockhart and Gresser 1971a, 1971b). There is no evidence to suggest that these alterations arise from any change in the induction requirements or post-translational events. The evidence does suggest that both amounts of transcription and translation are altered. Direct evidence for an increased amount of transcription has been obtained by assaying the amount of interferon mRNA made. An increased amount of interferon mRNA has been reported in cells pretreated before induction with 5'-bromodeoxyuridine and butyric acid (Morser et al , unpublished) and from superinduced cells (Seghal et al 1977, Reynolds, Prekumar and Pitha 1975 and Cavalieri et al 1979). In the latter case it seems that the increased transcription is not enough to account for the increase in yields of interferon made in intact cells (Raj and Pitha 1977 and Cavalieri et al 1977). Cycloheximide alone has also been reported to superinduce cells (Seghal, Dobberstein and Tamm 1977). These data suggest that superinduction is, at least in part, accounted for by increased translation. Priming cells with interferom gives rise to higher yields of interferon in response to an inducer without a detectable increase in transcription (Abreu, Bancroft and Stewart 1979). The production of interferon does however parrallel the amount of interferon mRNA detected in cells (Seghal et al 1977) suggesting that events prior to translation are rate limiting.

Two studies have shown that post-transcriptional modification of interferon mRNA occurs. It is known that interferon production declines to undetectable levels after reaching a peak rate. One reason for this could be that the interferon mRNA has a short half-life. It has been shown however that interferon mRNA is stable after injection into oocytes (Seghal, Lyles and Tamm 1978 and Colman and Morser 1979) and in metabolically blocked FS-4 cells (Seghal,

Tamm and Vilcek 1975), for several days. In the latter case the metabolic block can be reversed since both 5,6-dichloro-1-β-D-ribofuranosylbenzimadazole (DRB)- which inhibits RNA synthesis - and cycloheximide - a protein synthesis inhibitor - can be washed out. If this is done the rate of interferon production by the FS-4 cells rapidly declines to zero.

Alterations in the transcriptional modification of interferon mRNA have been proposed as a mechanism to account for the superinduction phenomena. Superinduction occurs in many systems and with a wide variety of metabolic inhibitors (Tomkins et al 1972, Chatterjee 1979). Three hypotheses have been put forward to account for superinduction. These are inhibition of degradation of the mRNA, a decreased competition for translation of the mRNA on polyribosomes, or an inhibition of inactivation of the mRNA.

In hepatoma cells superinduction of the enzyme tyrosine amino transaminase (TAT) is known. It is proposed that the levels of this and other intracellular enzymes must be tightly regulated. This may be achieved by having rapidly turning over mRNA's for these enzymes. Serum and insulin slow the enhanced degradation of TAT mRNA that occurs in growth inhibited cells. This results in a rise in the specific activity of TAT without a change in the rate of synthesis of the mRNA. Actinomycin D superinduces TAT in these cells and is thought to act by inhibiting degradation of TAT mRNA (Steinberg, Levinson and Tomkins 1975).

It seems unlikely that interferon mRNA would be regulated in this manner since interferon is actively exported from the cell (Tan,Jeng and Ho 1972), and not constituitively made.

Ovalbumin can also be superinduced by actinomycin D (Rhoads, McKnight and Schimke 1973). It is suggested that actinomycin D inhibits RNA synthesis as its primary site of action. This means that less stable cellular RNA's will be degraded faster than stable mRNA's. If ovalbumin mRNA is stable then there will be less competition for polyribosomes with time since the total mRNA population has declined. Thus a more efficient translation of stable mRNA's on polyribosomes will result, giving increased production of their protein products.

In the interferon system, actinomycin D treatment alone does not superinduce interferon (Tan and Berthold 1977). The fact that a number of inhibitors can be used to obtain superinduction (Tan et al 1970, Vilcek and Ng 1971, Seghal and Tamm 1975, Tan and Berthold 1977) supports the idea that the primary site of action of these inhibitors is a general inhibition of RNA and protein synthesis. This is in spite of the observations that certain inhibitors are not as specific as was at first thought. For instance, actinomycin D is used as an inhibitor of RNA synthesis but it has been shown to affect translation in vitro (Leinward and Ruddle 1977). The concentrations of both actinomycin D and cycloheximide used to give superinduction are also above those required for optimal inhibition of RNA and protein synthesis (Seghal, Tamm and Vilcek 1976). Superinduction can also be achieved with DRB and cycloheximide. DRB has been shown to inhibit mRNA synthesis including interferon mRNA (Seghal, Tamm and Vilcek 1976). Therefore it is not a preferential inhibition of other classes of mRNA leading to decreased competition of interferon mRNA for polyribosomes that account for the superinduction of interferon. If the DRB treatment is delayed until late in the 'shut-off' phase of interferon production, then interferon production declines for a further 3-4 hours and then stabilises for a prolonged period. If the interferon mRNA was simply displaced from the polyrobosomes during the 'shut-off' phase then it would be expected that a rise in interferon production would eventually be seen after such DRB treatment (Seghal and Tamm 1976). Furthermore, no translatable interferon mRNA can be extracted from cells that is not present on polyribosomes (Seghal, Dobberstein and Tamm 1977). Thus it seems that a simple competition model will not suffice to explain interferon superinduction.

Lastly it has been suggested that a repressor system operates which inactivates specific mRNA (McAuslan 1963). It is proposed that antimetabolites differentially inhibit the formation of this repressor (Tan et al 1970, Vilcek and Ng 1971). The model, as presented for interferon, is as follows (see figure 1).

The initial event - induction - is proposed to alter the equilibrium. Double stranded RNA is assumed to complex the repressor

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Figure 1.

The repressor hypothesis.

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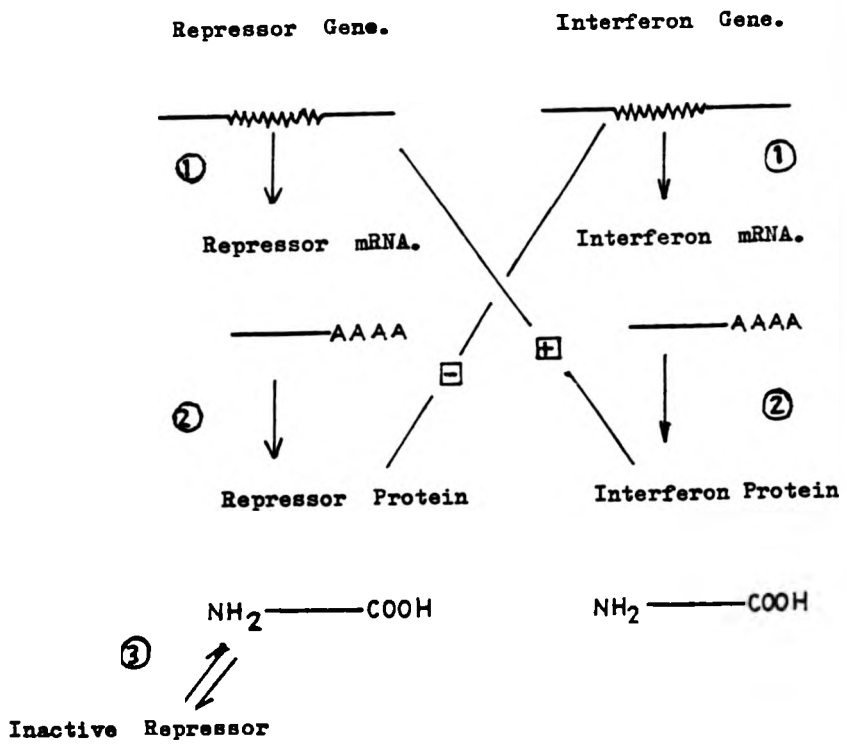


(Tan & Berthold 1977).

protein in some manner so as to inactivate it. This removes the repressor from the system and thus the negative control on the interferon gene. The gene is thus 'derepressed'. Interferon mRNA is then made and translated. Interferon itself is assumed to exert a positive control over transcription of the repressor gene. This system thus explains the 'shut-off' mechanism.

The use of antimetabolites 'selectively' alters this system. Cycloheximide is first added during or before induction. This acts at 2 in the diagram. Since no interferon can be made there is no transcription of the repressor gene. Interferon mRNA levels thus build up. Actinomycin D is then added which irreversibly inhibits RNA synthesis, so no further mRNA is made. Actinomycin D thus acts at 1 in the diagram. On removing the cycloheximide block by washing the interferon mRNA can be translated to interferon but repressor mRNA cannot be made so no repressor is made, in theory. However, a small amount of RNA synthesis still occurs in the presence of actinomycin D and therefore some repressor is made. The system is thus eventually 'shut-off' but interferon will have been made quicker than normal since there was a higher concentration of interferon mRNA at the onset of translation, and it will also have been made for longer because the repressor is made later and more slowly. Thus more interferon is made than in untreated cells. This model is based heavily on the Jacob and Monod model and therefore must be regarded with certain reservations as explained earlier.

Other observations on superinduction.

Only systems that are inducible with poly(rI).poly(rC) or UV-NDV can be superinduced. When wild type NDV is used only the early interferon response (i.e. interferon produced soon after induction) can be superinduced. This however only occurs when DRB is used (Vilcek and Kohase 1977) and not when actinomycin is used (DeClerq and Merigan 1970). In the latter case, as with the late response when using DRB, the interferon yields are depressed. This has been generalised to the statement that only systems that show an early response can be superinduced (Vilcek, Barmaks and Havell 1972).

Chick cells (Long and Burke 1971) and mouse L cells (Stewart, Gresser and Lockhart 1971) are reported not to be superinducible, whereas rabbit kidney cells are (Vilcek 1970); poly(rI).poly(rC) being used in all cases. It is suggested that the induction conditions rather than the control mechanisms differ. The only evidence to support this view comes from work done with mouse-human hybrid cell lines. Some of these cells make both human and mouse interferon, but the proportions of the two vary greatly depending on the inducing conditions. This suggests that the events leading to triggering may be different for different inducers. Both types of interferon could be superinduced, except in one hybrid where superinduction did not occur for either interferom (Frankfort et al 1978, Meager et al 1979). Higher doses of poly(rI).poly(rC) or the use of DEAE-dextram with the poly(rI).poly(rC), increases the uptake of the inducer (Tovell and Colter 1967). It is possible that higher doses of inducer neutralises more repressor and thus prevents an early 'shut-off'. If excess inducer is present then this will neutralise all the repressor and the addition of antimetabolites cannot make any difference to the interferon yield.

The state of repression of the interferon gene in a given type of cell may also determine the quantity of interferon produced. If the gene is poorly repressed then it will be easily induced and vice versa (Tan and Berthold 1977).

The repressor.

The identity of the repressor is not known but it is assumed to be a protein. It has been suggested that it is an interferon oligomer since this would explain the positive control of interferon over the repressor (Kleinschmidt 1972). However there is no direct evidence to support this idea.

The fact that a single metabolic inhibitor can give rise to small interferon yields in the absence of an inducer suggests that the repressor is very labile (Tan and Berthold 1977).

The repressor is involved in regulation in two ways. Firstly it could have a role in induction. Three hypotheses have been put

forward for induction. These are the double-stranded RNA hypothesis where double-stranded RNA formed in or taken up into the cell is the inducer, which exerts positive control over the interferon gene: the repressor depletion hypothesis ((3) figure I), where repressor is inactivated by the inducing molecule and negative control is thus removed; and the basal level interferon hypothesis, where low levels of constitutively made interferon exerts a feedback control over the expression of the interferon gene. The latter hypothesis is linked to the first two if one assumes that the repressor is interferon. Certain cells have been shown to produce small amounts of interferon in the absence of an identifiable inducer (DeMaeyer-Guignard, Thang and DeMaeyer 1977). It has thus been suggested that interferon has a cellular function other than its antiviral function. Interestingly, interferon can itself bind to many inducers including poly(rI) and poly(rU) as well as double-stranded RNA. This is a curious property since the binding appears to be very specific. The hypothesis thus states that the role of the inducer is to bind the basal levels of interferon normally made by the cell and that this induces further interferon synthesis.

Secondly, the repressor inactivates interferon mRNA. This could occur by two methods. The repressor could be a highly specific nuclease. Alternatively, the repressor could complex with the mRNA in such a manner as to prevent its translation (Vilcek and Ng 1971). The inactivation is irreversible since interferon mRNA activity cannot be rescued by ultraviolet irradiation of cells in the 'shut-off' phase. This treatment would be expected to inhibit repressor synthesis (Vilcek, Havell and Kohase 1976).

The repressor has been used to explain other observations. These are hyporesponsiveness, ultraviolet superinduction by preirradiation of the cells, and certain constituitive mutants.

It is unlikely that interferon itself is involved in hyporeactivity for the following reasons. Hyporeactivity can occur as soon as one hour post-induction (Breinig, Armstrong and Ho 1975). Hyporeactivity factors can also be separated from antiviral activity and cannot be neutralised with anti-interferon serum (Stringfellow 1975, 1977). Although high doses of interferon do

inhibit its own production (Vilcek and Rada 1962) it is also seen that low doses enhance its own production (Stewart, Gresser and Lockhart 1971). Cells can also show hyporeactivity to doses of endotoxin that do not induce any detectable interferon (Kleinschmidt 1972).

Induction is a required event for hyporeactivity - low doses of poly(rI).poly(rC) that do not induce interferon do not give hyporesponsiveness to subsequent higher doses of inducer (Billiau 1970, Breinig, Armstrong and Ho 1975). Thus it has been suggested that the repressor is involved (Billiau 1970, Tan et al 1970). More precisely it has been suggested that the ability of the repressor to irreversibly inactive interferon mRNA is the cause of hyporesponsiveness (Kohase and Vilcek 1977). To account for the rapid onset of hyporeactivity, it is suggested that repressor synthesis could be directly stimulated by the inducer, rather than by interferon (Breinig, Armstrong and Ho 1975). Hypo-responsiveness is a very confused field with many contradictory results appearing in the literature. These results may reflect different amounts of repressor being synthesized in different tissues (Ke and Ho 1971).

Ultraviolet irradiation (UV) of cells before induction gives rise to a superinduction effect (Linder-Frimmel 1974, Mozes et al 1974). Where poly(rI).poly(rC) is used as inducer and anti-metabolites used as well as ultraviolet pre-irradiation, then no increase over normal 'superinduction' yields is obtained. No superinduction is seen with NDV induction and ultraviolet pre-irradiation (Memes and Vilcek 1974). Thus UV superinduction could occur by an inhibition of repressor synthesis as a result of an inactivation of the repressor gene. Where a superinduction regime with antimetabolites is followed production may be fully expressed and the repressor system fully depressed. Thus UV treatment would have no further effect. Indeed interferon yields fall due to the action of the UV irradiation on the interferon gene(s). In the case of viral induction, it is suggested that superinduction does not occur because of the different inducing conditions resulting from a different induction site and/or triggering sequence (Vilcek,

Barmaks and Havell 1972), as discussed above.

Finally, selection procedures or mutagenesis have produced phenotypes that either continuously make small amounts of interferon (Jarvis and Colby 1978), or that make significantly higher amounts of interferon after induction (Tan et al 1979). These phenotypes could be the result of a malfunctioning repressor system.

Translation of interferon mRNA.

Interferon mRNA can be translated in intact heterologous cells, cell-free systems and in oocytes. The oocyte system is capable of relatively efficient translation of exogenous mRNA's, whereas the other two systems yield relatively small amounts of interferon. Cell-free systems are apparently inefficient at translating human interferon mRNA's probably because they cannot modify the resultant protein (Stewart 1979). Post-translational modification may be required for biological activity as is generally the case with the peptide hormones e.g. insulin (Butcher, Robinson and Sutherland 1972). Mouse interferon mRNA can be translated as efficiently in vitro as in intact cells (Montagnier et al 1974). The reason for this discrepancy between the two mRNA's is not known.

Translation of interferon mRNA's required spermine at critical concentrations (Montagnier et al 1974, Pestka et al 1977).

Post-translational control.

There is a lag of 20-30 minutes before intracellular interferon can be detected in the extracellular medium (Tan et al 1971, Ng, Berman and Vilcek 1972). It is suggested that glycosylation and secretion occur in this period. The synthesis of active interferon but not the secretion of interferon can be affected by inhibitors of glycosylation* (Havell et al 1975). A smaller antiviral protein

*However production of active interferon is not totally inhibited. The effect of such inhibitors on interferon synthesis could result from the inhibition of the activity of other cellular proteins which do require carbohydrate for activity.

has been reported in L cells treated with these inhibitors, having a molecular weight of 16,000 compared to native interferon which has molecular weights of 22,000 and 38,000. The difference in molecular weight is proposed to be due to differences in the extent of glycosylation (Havell, Yamazaki and Vilcek 1977). Chick cell extracts, that do not possess antiviral activity, elicit antibody to chick interferon (German, Quero and Poindron 1971). Ehrlich ascites cell extracts incubated with interferon mRNA for 30 minutes before addition of sparsomycin to block further protein synthesis, do not show detectable antiviral activity until incubation has proceeded for another two hours (Pestka et al 1977). These results suggest that some kind of post-translational modification is required to give biologically active interferon.

Intracellular interferon is trypsin resistant, unlike extracellular interferon. It is suggested that intracellular interferon is bound inside vesicles (Ng, Berman and Vilcek 1972, Falcoff et al 1976, Morser et al 1979) and thus is protected from the protease. Extracellular interferon injected into oocytes is degraded or inactivated. This supports the idea that interferon is compartmentalised after translation (Morser et al 1979).

A rapid in vivo degradation of cytoplasmic glycoproteins has recently been reported (Kalish, Chovik and Dice 1979). They support the suggestion that glycoproteins are turned over faster than non-glycoproteins (Curd and Evans 1973, Olden, Pratt and Yamada 1978, Ashwell and Morrell 1974, 1975) but show that they are no more sensitive to pronase of chymotrypain. Thus interferon is not peculiar in this respect and glycosylation may be a signal to the cell for selective degradation. Interferon may be degraded rapidly to minimise its anticellular, or other effects, in the whole animal.

The secretion of interferon is an active process since it can be stopped at 4°C even though there are large amounts of intracellular interferon (Tan, Armstrong and Ho 1971, Field et al 1972). The involvement of microtubules and microfilaments are implicated in the production of interferon, since specific inhibitors of their formation inhibits the production of interferon (Ito, Nishiyama and Shimokata 1976). The precise function of microtubules and

microfilaments are not known but suffice to say that they are involved in many cellular and developmental processes (Wessells et al 1971).

A specific system for the production of interferon.

Lymphoblastoid cells, and the Namalwa line in particular, produce high titres of interferon in response to infection with negative stranded RNA viruses (Strander, Mogenson and Cantell 1975, Volckaert-Vervliet and Billiau 1978 and Zoon et al 1978). It is not known why these cells make higher titres of interferon than most other cells but the controlling property could lie with the transforming viral genetic information that most of these cell lines carry, with the host regulatory mechanisms or with the inducer itself. Let me consider these points further.

Lymphoblastoid cells.

Lymphoblastoid cells can be established from a variety of sources, including healthy individuals. They are lymphocyte-derived and grow in suspension culture for an indefinite period. These cells can be sub-classed according to their origin and their possession of viral gene sequences. Cells derived from patients suffering from infectious mononucleosis or Burkitts Lymphoma generally contain part or all of the Epstein-Barr virus genome. The latter cells are referred to as lymphoma cells (Nilsson and Ponten 1975). Lymphoblastoid cells make varying amounts of interferon either spontaneously (Adams, Strander and Cantell 1975) or after induction which is generally done with paramyxoviruses (Strander, Mogenson and Cantell 1975). These cells are similarly differentially sensitive to the antiviral and anticellular effects of HLeIF (Hilfenhaus, Damm and Johannsen 1977).

Epstein-Barr virus.

Epstein-Barr virus (EBV) was discovered in lymphoma cell lines established from Burkitts Lymphoma (Epstein and Barr 1964). It is

a member of the Herpes group of viruses but apart from transforming certain cell types the virus shows very little biological activity *in vitro*. The role of EBV in the formation of Burkitts Lymphoma is not yet known but it seems that there must be certain cofactors (Gunven 1975, zur Hausen 1975, Ablashi 1976, Ponten 1976, Rapp and Reed 1977 and Henle, Henle and Lennette 1979). It is, however, the cause of infectious mononucleosis and appears to be involved in another type of human cancer-nasopharyngeal carcinoma.

Two types of EBV can be identified *in vitro*. One type is transforming and the other is non-transforming. The former is known only to be made by a single cell line, is a heterogeneous population of virus (Fresen *et al* 1972) and lacks some of the genetic information of the non-transforming virus (Given and Kieff 1978). Progeny virus with the properties of both viruses can be obtained (Traul *et al* 1977). The virus exists both in a circular form (Lindahl *et al* 1976) and in an integrated form, both having alkali labile bonds suggesting that RNA linkers may be involved (Adams, Lindahl and Klein 1973). The integration of the viral genome appears to be random (Spira *et al* 1977). The transforming strain occurs in circular forms that are smaller than the non-transforming virus (Adams *et al* 1977) and it is possible that they arise by the loss of one of the two homologous reiterated terminal sequences present in the non-transforming viral genome. Each lymphoma cell line possesses a different number of EBV genome equivalents but the number in a given line remains constant even in cell lines that do not produce progeny virus (Schwarts, Cook and Harris 1971).

It is assumed that transformation of the lymphoma cells by EBV is responsible for their 'immortalisation'. All cells of a given cell line contain EBV (Miller 1970, Maurer *et al* 1970 and Zajac and Kohn 1970). Few cell lines produce EBV and some produce only one EBV-related antigen - Epstein-Barr nuclear antigen (EBNA). The possession of this antigen is absolutely correlated with the possession of EBV genetic material (Anderson and Lindahl 1976). Thus it seems likely that only a few genes are required to maintain transformation (Hayward and Kieff 1976).

The EBV genome is about 10^8 molecular weight DNA and could

potentially code for about 100 proteins (Prichett, Hayward and Kieff 1975), of which 33 have been identified on polyacryamide gels (Dolyniuk, Prichett and Kieff 1976). Thus it seems that in lymphoblastoid cells, expression of the viral genome is repressed. There is evidence to support this idea. Firstly, cells in a biopsy of Burkitts Lymphoma rarely produce viral entigens, but once established in culture such expression often occurs, although in only about 1-4% of the cells (Zajac and Kohn 1970). This expression can be increased by culturing the cells in the absence of arginine (Henle and Henle 1968). Secondly, latency occurs *in vivo* without any clinical symptoms, although it is not known what host factors govern the course of an EBV infection *in vivo* (Ablashi *et al* 1976). Thirdly, the evidence from cell-hybrids suggests that an inhibitor is present that prevents a productive infection. This inhibitor can be antagonised to allow other viral antigens to be produced (Nyormoi *et al* 1973). Treatment of the cells with thymidine analogues, particularly bromodeoxyuridine, allows the production of viral antigens and a productive cycle, possibly as a result of mutation (Gerber *et al* 1972 and Hampar *et al* 1973). The phenotypes of fibroblast/lymphoma cell hybrids argues against a virally produced inhibitor (Klein *et al* 1976). Cell surface marker expression also appears to be under host control (Robinson 1977). The inhibition acts at a post-transcriptional level, since iododeoxyuridine treatment of Raji lymphoma cells increased the transcription of EBV DNA from 25% to 50% but no further expression of viral entigens was observed. In hybrid cells such treatment gave rise to new antigen expression (Tanaka *et al* 1979).

In the light of the observation that certain lymphoma cell lines make interferon autogenously, it has been suggested that this interferon acts as the negative control element inhibiting EBV expression (Adams, Strander and Cantell 1975). In view of the fact that leucocytes that are not infected with EBV and other lines established that do not contain EBV genetic material make interferon in response to an inducer (Strander and Cantell 1966), it seems unlikely that EBV plays a determining role in the expression of interferon. However the mechanism of production of autogenous

interferon is unknown. This interferon is identical to virally induced interferon, is produced by all the cells in a given line and enhances the rate of production of virally induced interferon (Zajac, Henle and Henle 1969).

The Namalwa line of lymphoma cells.

Namalwa is a lymphoma cell (Klein, Dombos and Gothoskar 1972) which grows in suspension, has no known enzyme deficiency, makes immunoglobin M lambda protein and has a model chromosomal number of 45. It contains EBV DNA, which may be a defective genome (Prichett, Pederson and Kieff 1976). In addition to hybrid work, which shows that Namalwa cells contain an EBV repressor (Nyormoi et al 1973), work on RNA sequences shows that only about three EBV proteins are made (including EBNA). These three proteins must be sufficient to maintain transformation. There is regulation of transcription from EBV sequences and a selective exclusion of some transcribed sequences from stable polyribosomes (Hayward and Kieff 1976).

Namalwa cells make both autogenous interferon (Adams et al 1975) and virally induced interferon (Strander, Mogenson and Cantell 1975). The interferon is of leukocyte type, although a small proportion is specifically neutralised by anti-fibroblast interferon anti-serum (Havell, Yip and Vilcek 1978). It is suggested that these two antigenically different species of interferon have separate structural genes (Cavalieri et al 1977). Namalwa cells, however, appear to be insensitive to leukocyte interferon in that the antiviral state is not achieved (except for VSV and EBV where other host factors may be involved, Lidin and Adams 1975, Nowakowski et al 1973). Namalwa cells are sensitive to relatively high levels of interferon in terms of growth inhibition (Volckaert-Vervliet et al 1978). Recently the optimal conditions for the production of interferon by Namalwa cells have been studied (Zoon et al 1978, Johnston et al 1979).

Sendai virus - the virion and its replicative cycle.

Sendai virus is a negative stranded RNA virus classified as a

paramxyovirus and otherwise known as HVJ virus or parainfluenza I. The virion is approximately spherical consisting of an envelope containing lipid surrounding a helical nucleocapsid, which contains single stranded RNA of about 6 x 10^6 molecular weight. Spikes containing the virus specific haemagglutinin and neuraminidase proteins protrude from the envelope. The virus replicates in the cytoplasm of a permissive cell with virus components being assembled at the cell membrane. The virus is released by budding.

The RNA represents 3.7% of the weight of the nucleocapsid, sediments of 50S in 0.1M NaCl and has a buoyant density of 1.680 in CsCl. The virus contains a virus specific RNA dependant RNA polymerase (Robinson 1971) which produces 18 and 22S complementary RNA (Blair and Robinson 1967). Some 50S RNA in the virions is message sense (Robinson 1970). Passage of the virus at high multiplicity in eggs gives rise to the generation of incomplete genomes (Kingsbury and Portner 1970).

Thirteen different defective genomes have been identified (Kolakofsky 1979) ranging from 4-47% of the wild type genome. Each strain of Sendai is predisposed to generate a specific RNA and shorter RNA's are not selectively amplified over the larger ones. The mechanism of generation of these particles and the nature of the advantage they have over the wild type genomes are not known. Passage of Sendai is thus normally carried out at low multiplicity.

The haemagglutining activity of the egg passaged virus is variable from passage to passage whilst infectivity is retained. This is attributed to changes in the envelope such as permeability changes (Homma et al 1976). Sendai virus passaged in tissue culture is generally non-infectious. The progeny virus contain a precursor glycoprotein termed Fo which is normally cleaved in eggs to form proteins F1 and F2 by breakage of two disulphide bond linked chains (Schied and Choppin 1974). Tissue culture grown virus can be made infectious by treating with trypsin, which cleaves the Fo protein to F1 and F2 (Schied and Choppin 1977). Non-infectious virus retains full haemagglutining and neuraminidase activity but not cell fusing ability and thus F1 and F2 are implicated in the fusion process (Schied and Choppin 1974). The non-infectious particles cannot be activated by a permissive cell line and so the host protease responsible for cleaving Fo must be intracellular (Pennington 1978).

Sendai virus possesses four other structural proteins plus two minor components. These are the nucleoprotein (NP), L and P proteins found in the necleocapsid and possibly involved in the RNA dependant RNA polymerase activity, and the matrix (M) protein (Schied and Choppin 1977). The two minor components have molecular weights of 54000 and 42000, the former being related to NP and the latter being cellular actin (Lamb and Choppin 1978). L is a large protein but does not represent a precursor protein since it has a unique peptide map (Emerson and Yu 1975). There are two non-structural polypeptides one of which is a phosphorylated form of the M protein, while the other is unrelated to any of the structural proteins (Lamb and Choppin 1977b, 1978). The intracellular distribution of these polypeptides has been described (Lamb and Choppin 1977a).

The M protein may be involved in the binding of the nucleocapsid to the envelope during maturation (Schimizu and Ishida 1975). It is also involved in the formation of restricted areas of plasma membrane which contains viral glycoproteins and thus gives rise to the viral envelope (Yoshida *et al* 1979). The haemagglutinin protein is a glycoprotein but is not involved in virus assembly. It appears to be an important factor in cell killing (Portner *et al* 1975).

Role of Sendai virus in interferon induction.

Sendai virus is the best inducer of interferon known giving high titres of interferon after infection of Namalwa cells (Strander, Mogenson and Cantell 1975, and Johnston *et al* 1979). Sendai presumably induces as a result of its ability to form double-stranded RNA replicative intermediates.

Lymphoblastoid interferon.

Lymphoblastoid interferon is mostly leukocyte-type interferon (see page 9). Thus since most of the clinical applications of interferon to date have utilised HLeIF (Pollard and Merigan 1979), lymphoblastoid cells could provide a valuable source of further interferon to build on past data. Fibroblast interferon has not been

used a great deal because of the problems involved in handling large volumes of monolayer cells and the low titres of interferon generally obtained. The fact that lymphoblastoid cells grow in suspension and have an indefinite lifespan (Nilsson and Ponten 1975) is an obvious advantage in large scale work. Fibroblast interferon also seems to give more severe side reactions at the site of injection than leukocyte interferon (DeSomer et al 1977). Other advantages of Namalwa cell interferon are the fact that high titres of interferon can be produced in cultures without a requirement for serum (Zoon, Bridgen and Smith 1979). This makes purification somewhat easier and production cheaper.

The main problem with lymphoblastoid interferon is that preparations may contain not only the inducing virus but also genetic information from the Epstein-Barr virus residing in the cell. The former is sensitive to low pH and extensively inactivated. The latter is of particular importance since it is the only virus for which there is any substantial evidence for a role in human oncogenesis. As the genome is large and the biological roles of the polypeptides that can be identified in any infected cell are not known it will be important to make sure that no EBV related material is present in the final preparations. The best way of achieving this in the long term will be to isolate the interferon gene alone and sequence it in comparison to the protein sequence. If this can be inserted into bacterial cells, then no EBV expression can occur. Genetic manipulation also offers other advantages as previously discussed. The technique of DNA-DNA hybridisation using EBV complementary DNA, prepared from purified EBV DNA, shows no hybridisation with partially purified preparations of interferon from lymphoblastoid cells (Lindahl et al 1976). Thus one can be reasonably sure that EBV genetic material is not present in these preparations of interferon.

The initial case for the use of interferon is in cases where no other treatment is available (e.g. Hepatitis B), so the advantages of treatment with lymphoblastoid interferon must outweigh the disadvantages.

Materials and Methods.

Chemicals.

DABA (3',5 - Diaminobenzoic acid dihydrochloride) (Gold Label) was purchased from the Aldrich Chemical Co. Ltd., Gillingham, Dorset.
Acrylamide; Ammonium persulphate; ferric ammonium sulphate; Folin and Ciocalteau reagent; n-butyric acid; sodium dodecyl sulphate; especially pure grade (SDS) and sodium pyruvate, were purchased from B. D. H. Chemicals Ltd., Poole, Dorset.
Caesium chloride (ultra pure reagent) was purchased from Bethesda Research Laboratories, Rockville, Maryland, U.S.A.
5'-Chlorodeoxyuridine (CldUrd) was purchased from Calbiochem Ltd., London.
N,N^1-Methylene bisacrylamide and N,N,N^1,N^1-tetramethylenediamine (TEMED) was purchased from Eastman Kodak Co., Rochester, New York, U.S.A.
Perchloric acid (70%) was purchased from Fisons Scientific Apparatus, Loughborough.
Whatman GF/C filters were purchased from Griffin and George Ltd., London.
2,5-Diphenyloxazole (PPO) and 1,4-di (2 (5-phenyl-oxazolyl))-benzene (POPOP), both scintillation grade, were purchased from Nuclear Enterprises (GB) Ltd., Edinburgh.
Soluene 350 was purchased from Packard Instrument Co. Inc., Illinois, U.S.A.
Ficoll was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.
Glutaraldehyde, osmium tetroxide and uranyl acetate, were purchased from Polaron Equipment Ltd., Watford.
5'-Bromodeoxyuridine (5'-BrdUrd); deoxyribonucleic acid (DNA)- from calf thymus; 5'-fluorodeoxyundine (FdUrd); 5'-iododeoxyundine (IdUrd); 2-mercaptoethanol, type 1; ribonucleic acid (RNA), type from yeast and trizma base (tris (hydroxy-methyl) aminomethane) were purchased from Sigma Chemical Co., London.

Lead citrate and Spurrs Resin kit for electron microscopy were purchased from Taab Laboratories, Reading.
All other materials were the best grade available commercially.

Radiolabelled compounds.

The following were purchased from the Radiochemical Centre, Amersham, Bucks.

L - ^{35}S methionine	745 - 880 Ci/mmol.
86 - Rubidium	1 mCi/ml.
5 - ^{3}H thymidine	42 - 47 Ci/mmol.
5 - ^{3}H uridine	12 - 28 Ci/mmol.

Biological materials.

Noble agar and tryptose phosphate broth were purchased from Difco Laboratories, East Molesley, Surrey.
All media and serum were purchased from Flow Laboratories Ltd., Irvine, Ayrshire, Scotland.
Crystamycin (containing 600mg (10^6 units) of pencillin to 1g of streptomycin) was purchased from Glaxo Laboratories Ltd., Greenford, Middlesex.
Bovine serum albumin (fraction V), cycloheximide, DEAE dextran, N-acetyl trypsin, sodium pyruvate and trypsin type III (from bovine pancreas) were purchased from Sigma Chemical Co., London.
Adult chicken red blood cells were purchased from Tissue Culture Services.

Tissue culture medium.

All media were supplemented with 100 units/ml crystamycin.
Bovine turbinate cells (BT). Growth medium - GMEM supplemented with 10% (U/V) horse serum.
Maintenance medium GMEM supplemented with 2% (V/V) horse serum.
Human foreskin fibroblasts (HFF). Media identical to that used for BT cells but horse serum replaced by foetal calf serum.

Malin-Darby Bonne Kidney cells (MDBK). Media identical to that used for Namalwa cells except that foetal calf serum was used instead of newborn calf serum.

Namalwa cells. Growth medium - RPMI 1640 supplemented with 10% (V/V) newborn calf serum.

'Maintenance' medium. RPMI 1640 supplemented with 2% (V/V) newborn calf serum.

Primary chicken embryo fibroblasts prepared by the method of Morser, Kennedy and Burke (1973) were maintained in 199 medium +2% newborn calf serum.

Buffers and solutions.

Alsevers buffer was made up as follows:-

D-glucose	20.5 g/l.	
Na Cl	4.2 "	pH 6.1 - 6.2.
trisodium citrate dihydrate	8.0 "	
citric acid	0.55 "	

Bacto agar was made up 2% (wt/v) in distilled water and autoclaved at 15psi for 15 mins. immediately before use and maintained at 42°C. Noble agar was made up at 1.8% (wt/v).

DEAE dextran was made up 5% (wt/v) in PBS and stored at 4°C.

Cacodylate butter. 50ml 0.2M sodium cacodylate ($Na(CH_3)_2AsO_2\ 3H_2O$) in distilled water plus 2.7ml of 0.2M HCl made up to 200ml with distilled water.

N acetyl trypsin was made up at 2mg/ml in PBS and frozen at -20°C in 300ul aliquote.

Phosphate buffered saline (PBS) was 139mM NaCl containing 28mM KCl, 7.5mM Na_2HPO_4 and 0.147mM KH_2PO_4 (pH 7.4) sterilised by autoclaving at 15psi for 15 mins.

pH 2.0 buffer. 6.5ml 2M HCl in distilled water plus 43.5ml 2M KCl in distilled H_2O made up to 1 litre with distilled water.

TNE was 50mm tris containing 100mm NaCl and 1 M ethylene diamine tetra-acetic acid (disodium salt) (pH 7.4) made up to 20 fold concentrate in distilled water and autoclaved at 15psi for 15 mins. before used.

Trypsin for monolayer cell passage was made up as follows to give a 50 fold concentrated stock.

Trypsin (type III from bovine pancreas)	4mg/ml.
disodium EDTA $2H_2O$.	4mg/ml.
Na Cl.	7.6mg/ml.
K Cl.	224 µg/ml.
Glucose.	1.8mg/ml.
$Na_2 HPO_4$ (anhyd).	142 µg/ml.

The solution was adjusted to pH7.0 by addition of sodium hydroxide (IM), sterilised by filtering through a 0.22µm filter (Millipore S.A. Molsheim, France) and stored at -20°C.

Trypsin for use was diluted to 1x concentration with PBS to give a final concentration of 80µg/ml.

Scintillation mixtures.

Toluene scintillation mixture was 4g PPO per litre of toluene.

Acidified toluene scintillant was supplemented with 1ml per litre of glacial acetic acid.

Triton-toluene scintillation mixture was 1 volume of Triton X-100 and 2 volumes of toluene scintillation mixture containing 0.266g/l final concentration of POPOP.

Electron microscopy - Spurrs resin.

The resin from the Taab Spurrs resin kit was made up immediately before use as follows:-

Epoxy resin	ERL 4206 (vinyl cyclohexene dioxide)	10.0g.
Hardener	NSA (nonenylsuccinic anhydride)	26.0g.
Flexibiliser	DER 736 (diglycidyl ether of polypropylene glycol)	6.0g.
Accelerator	5-1 (dimethyl aminoethanol)	0.4g.

The first three ingredients are swirled to mix before the 5-1 is added. After shaking to disperse the accelerator the resin is ready to use.

Methods.

Growth of viruses.

Semliki Forest virus (SFV) stock was a plaque purified virus originally obtained from Dr. J. Porterfield, National Institute of Medical Research, Mill Hill, London.
Primary stocks were passaged in the brains of suckling mice (Walters, Burke and Skehel 1967).
Secondary stocks, which were used for experimentation, were prepared from suspension cultures of chick embroy cells using the modification of the method of Zwartow and Algar (1968) as described by Kennedy and Burke (1972).
Clarified supernatants supplemented with 5% (v/v) newborn calf serum from the latter procedure were stored at -70°C until use.
Sendai virus (HVJ) was a gift from Dr. N. B. Finter, Wellcome Research Laboratories, Beckenham, Kent. The virus was maintained by passage of dilute virus supplemented with 1mM Ca^{2+} and 1mM Mg^{2+} ions, in the allantoic cavity of 10 day old embroyonated eggs in the manner of Kingsbury and Portner (1970).
Allantoic fluid was collected 60 hours after innoculation from chilled eggs and clarified by centrifugation (2,000g for 10 mins.). The supernatant was frozen in aliquote in an ethanol: dry ice bath and stored at 70°C.
Subculture of cells.

Suspension cells. Namalwa cells obtained from Wellcome Laboratories, Beckenham, Kent, were routinely passaged every 2-4 days by dilution of the culture with growth medium until the cell density was about 0.5×10^6 cells/ml. Occasionally cultures were additionally buffered with 2M NaCH. Incubation was at 37°C.
Monolayer cells. Bovine turbinate (BT) cells were a gift from Dr. A. W. McClurkin, National Animal Disease Centre, Ames, Iowa, U.S.A.
HFF cells were derived from human foreskin fibroblasts and were a gift from Dr. T. Merigan, Stanford University.
Malin-Darby bovine kidney cells (MDBK) were a gift from Dr. Appleyard, Wellcome Research Laboratories, Beckenham, Kent.

BT, HFF and MDBK cells were passaged in their respective growth media using the following protocol. Monolayers of cells were washed 3x with 50ml PBS to remove serum. 5-10ml trypsin solution was added and the cells incubated at 37°C. Cell clumps were broken up by pipetting and dispersed to flasks for appropriate dilution (dependant on growth rate) in growth medium. Except for flasks containing MDBK cells, the flasks were gassed to 5% (v/v) with CO_2 and incubated at 37°C, Chicken embroyo fibroblasts were used as primary cultures prepared from 11 day old embryonated eggs and not suboultured. These cells were seeded in 199 medium plus 5% newborn calf serum at 10^7 cells/5cm petri dish and incubated for 24 hours at 37°C. Thereafter cells were maintained in maintainence medium until used. CEF cells were incubated at 37°C in an 95% air/5%CO_2 atmosphere.

Determination of cell number was done using an improved Neubauer counting chamber with cells suitably diluted with PBS and mixed 1:1 with PBS containing 0.1% trypan blue. Only cells excluding the dye were considered viable and counted.

Induction of interferon. Unless otherwise stated the induction of interferon in Namalwa cells with Sendai virus was as follows:- Namalwa cells were centrifuged from growth medium (2,000g for 5 mins.) and resuspended in maintainence medium at 1.0 x 10^6 cells/ml. 100 HA of Sendai virus per ml (i.e. per 10^6 cells) was added and the cells incubated for 24 hours at 37°C. The cells were pelleted from the medium by centrifugation (2,000g for 5 mins.) and the supernatant dialysed against pH2 buffer at 4°C for 24 hours or longer. Where necessary the dialysate was returned to neutral pH by dialysing against cold PBS for eight hours or more.

Induction with deoxycytidine.

Deoxycytidine HCl in PBS adjusted to pH7 with NaCH was used in the pretreatment medium in the presence or absence of 5'-BrdUrd where indicated.

Induction with butyric acid.

The conditions given here were those used by Dr. N. Johnston of Wellcome Research, Beckenham (personal communication).

Induction with thymidine and its analogues:

5'-BrdUrd, CldUrd, FdUrd and IdUrd.

The same induction as normal with conditions altered where specified. Pretreatment was with 25µg/ml of 5'-BrdUrd in PBS (or equivalent (81.4mM) of other analogues or thymidine itself) in the normal medium for 48 hours, unless otherwise specified.

Note that unless specifically stated none of these chemicals were present during the induction and production of interferon, since the cells were removed prior to induction by centrifugation and replaced in maintenance medium which was not supplemented with these chemicals.

Assay for interferon. The method used was the RNA reduction assay by Atherton and Burke (1975). Monolayer cells are grown in scintillation vial inserts (2 x ½ inch), being seeded at 1 x 10^5/ vial in growth medium and incubated at 37°C in 95% air/5% CO_2 until confluent. The medium is removed and dilutions of interferon in maintenance medium added to separate vials in a volume of 0.5ml/vial. A standard preparation of interferon calibrated against the 69/19 human leukocyte interferon standard (NIH, containing 5,000 reference research units/ml) was always included for every tray of 200 vials. Vials without interferon are also included. After overnight incubation the interferon is removed and the monolayer cells challenged with Semliki Forest virus at a 1 in 20 dilution from stock in maintenance medium supplemented with 1µg/ml actinomycin D and using 0.2ml/vial (about 50 to 100 pfu/cell). Controls for non interferon treated infected cells and uninfected cells are included. After 2.5 hours incubation at 37°C, ^3H uridine (1µCi/vial) was added in 0.2ml maintenance medium for a further 2.5 hours. Incorporation into virus specific RNA will occur in the presence of actinomycin D. Incorporation is terminated by removal of the medium and addition of cold 5% TCA. The monolayers are washed with cold 5% TCA and cold ethanol and dried at 60°C. 0.2ml 'Soluene' (diluted 1:30 in toluene) is added to dissolve the monolayer and subsequently 3ml of acidified toluene scintillant added. Vials were counted for radioactivity in a Packard 3385 at 70% gain. The titre of interferon is determined as the $\log_{10}$ dilution at which

incorporation of ^{3}H uridine into virus specific RNA is reduced by 50% compared to non interferon treated cells. The standard interferon is included such that the titre obtained can be corrected to reference research units (denoted U/ml). Titres are quoted herein as U/ml/10^6 cells unless otherwise stated.

HA test for Sendai. The haemagglutinating properties of Sendai can be used as a quanititative assay for Sendai virus particles, though the assay detects all virions be they infectious or not. The virus is diluted one hundredfold and then every twofold using PBS as diluent. The virus is titred in 250µl, adding 25µl of 5% adult chicken red blood cells in alsevers solution. The last dilution at which the erythrocytes agglutinate is called the titre and converted to the titre/ml referred to as HA/ml.

Plaque assay: General method.

5cm Petri dishes containing monolayer cells were washed three times with PBS to remove serum and incubated with a dilution of virus. After a period of time the innoculum was removed and 4-5ml of maintainence medium containing a final concentration of 0.9% (wt/vol) Noble agar and 100 units/ml crystamycin placed over the cells and allowed to set. Plates were then incubated at 37°C in 95% air/5% CO_2 until stained with 0.025% neutral red in buffered Earles saline for 1 hour. Plaques were counted by eye.

For SFV. The monolayer cells were primary CEF's. The innoculum contained 0.075% (w/v) DEAE dextran to increase the plaquing efficiency and was 0.5ml. Incubation with the innoculum was 2 hours at 37°C. Incubation with overlay was 1-2 days.

For Sendai. The monolayer cells were MDBK cells. The medium used for dilution of the virus was RPMI 1640 without serum but containing 10% tryptose phosphate broth. The innoculum was 0.1ml/plate. Incubation was 1 hour at room temperature. The overlay medium was supplemented with 0.1% (wt/vol) DEAE dextran and 2µg/ml of N-acetyl trypsin (final concentrations) and 1% (wt/vol) bovine serum albumin.

Quantitation of SFV specific RNA. Namalwa cells were resuspended at 2.5 x 10^6/ml in RPMI 1640 + 2% NCS at 37°C, following centrifugation (2000g for 5 mins) from growth medium. At zero time actinomycin D (1µg/ml) was added to the cells. After 1 hour SFV was

added to half the culture at approximately 20pfu/cell. Samples from both cultures were then labelled for periods of 1 hour with 20μCi of ^{3}H uridine, incubated at 37°C. Incorporation of ^{3}H uridine was stopped by the addition of one volume ice cold PBS and centrifugation (2,000g, 5 mins).

Quantitation of radiolabel - general method.

TCA precipitable incorporation of radiolabel was determined by collection of the precipitate on Whatman GF/C filters by filtration. The folters are washed with 2 x 10ml ice cold 5% TCA, 2ml ice cold ethanol and 2ml ice cold acetone. The filters are dried under an infra red lamp and counted in a Packard Tri Carb 3385 in toluene scintillant. Gains used were 70% for tritium and 13% for sulphur-35. TCA soluble radioactivity was determined by counting a 250μl sample in 3ml of triton-toluene.

Incorporation of radioactive precursors into Namalwa cells.

Namalwa cells were resuspended in serum free RPMI 1640 prior to infection with Sendai virus. Subsequently the cells were labelled for 1 hour at 37°C with either 2μCi/ml of ^{3}H uridine or 2μCi/ml of ^{3}H thymidine. TCA precipitates were prepared as above. The TCA soluble supernatant was also sampled for radioactivity by placing 250μl into 3ml of triton-toluene scintillant and counting in the normal manner.

For ^{35}S methionine labelling the procedure was similar (2μCi/ml) except that cell samples were starved for methionine by incubating them in GMEM-methionine at 37°C for 30 mins. prior to the addition of label. TCA soluble and preciptable counts were collected as above.

Labelling with Rubidium-86.

The influx of Rb^{86} was determined by the addition of about 50μCi $Rb^{86}Cl_2(aq)$ to 100ml of Namalwa cells at 2 x 10^6/ml in RPMI 1640 medium without serum and incubated at 37°C. Efflux was determined by prelabelling Namalwa cells for 1.5 hours and determining radioactivity in the same manner as for influx.

Incorporation or loss of label was stopped by the addition of 2 volumes of ice cold PBS at 4°C at 2,000g for 10 mins. The cell pellet was taken up in 1ml cold TCA and counted for radioactivity in a Packard Tri Carb 3385 at 1.5% gain using triton-toluene scintillant.

Two dimensional gel electrophoresis.

Sample preparation.

Samples from Namalwa cells incubated with 250μCi ^{35}S methionine in GMEM without methione, were prepared as follows:-
Incorporation was halted by addition of ice cold PBS and the cells centrifuged out of the medium at 200°g for 5 mins. The pellet is washed with cold PBS and recentrifuged. Cold 5% TCA is added to the pellet and the precipitate collected by centrifugation at 5,000g for 10 mins. The precipitate is taken up in buffer B (0.01M Tris HCl pH7-4, 5mM $MgCl_2$ and 50μg/ml pancreatic RNase). A final concentration of 50μg/ml DNase is added and incubated on ice for at least five minutes. Urea is added to a final concentration of 9M. One volume of buffer A (9.5M urea, 2% (w/v) NP40, and 2% ampholines made up of 1.6% pH range 5-7 and 0.4% range pH 3-10, and 5% β-mercaptoethanol) is added and samples either run immediately on an isoelectric focussing gel or frozen at -20°C. Two dimensional electrophoresis was carried out by the method of O'Farrell (1975) using isoelectric focussing as the first dimension (pH 3.5-10) and 8-20% gradient polyacrylamide gel electrophoresis in the second dimension.

Chemical determination.

DNA was measured by the method of Setaro and Morley (1977). Namalwa cell DNA was prepared by precipitating whole cells with 20% TCA and then sonicated on ice. The precipitate was collected by centrifugation (5,000g, 10 mins.). The pellet was washed by 5% TCA and re-centrifuged. DNA was extracted by heating the pellet at 70°C in 0.5M perchloric acid (PCA) (in distilled water) for 15 mins. The precipitate was repelleted and fresh 0.5M PCA added and the extraction repeated. Both supernatants were combined and assayed with diaminobenzoic acid colourimetrically at 420mm in a Gilford 250 spectrophometer. Protein was determined by the method of Lowry (1951) using a sample of the initial TCA precipitate from above.

Cloning of Namalwa cells from single cells.

The basic method is the agar pearl technique (Hinamura and Grace 1968). Namalwa cells were grown at densities between 1 and 2 x 10^5/ml for two weeks, never reaching densities greater than 5 x 10^5ml to select for better growth prior to cloning. Noble agar (Difco) 2% or agarose (Sigma), 2.0% in distilled water, were used freshly autoclaved and at a final concentration of 0.4% for plating in

microtitre plates or 5cm petri dishes (Flow). Glasgow modified minimal essential medium (GMEM) (Flow) was used at 10x concentration for the following mixtures:

1ml 2.0% agar at 42°C was added to a warm bijou containing:-

10µl sterile sodium pyruvate (500mM in distilled water).
100µl 10 x GMEM.
80µl 5% $NaHCO_3$ (in distilled water).
200µl foetal bovine serum (Flow).
3.6mls of cells in GMEM + 20% foetal bovine serum at an appropriate dilution.

Plates were overlayed with 2-5ml GMEM + 20% FBS and microtitre plates with 50µl of the same and incubated at 37°C, 5% CO_2 for about 2 weeks before colonies appeared.

Preconditioned medium was made from a 2:3 dilution of normal medium and the supernatant from cells grown for 48 hours in normal medium from a density of 4 x 10^5 cells/ml, and then filtered through a 0.22µ millipore filter. This was used to replace the standard medium as diluant and overlay for the cells in some experiments.

Colonies were picked with pasteur pipettes from the agar when visible under a Wild Heerdrugg Ltd. binocular microscope and transferred to microtitre plates with 200µl of RPMI1640 plus 20% FBS medium containing 1mM pyrivate and fresh glutamate and the agar dispersed by pipetting up and down in the well. Clones of cells that grew well after dispersal were transferred to bijoux with 1ml fresh medium and diluted further as determined by trypan blue determination of cell density.

Ultracentrifugation.

The buoyant density of cellular DNA was determined by centrifugation at 44,000 rpm in a Beckman model E ultra centrifuge run overnight to equilibrium. The density gradient used was caesium chloride (ρ = 1.400 g/cm) and a marker DNA from E.coli (ρ_m = 1.710 g/cm^3) was used for calibration of the gradient. The density of the DNA was determined using the formula $\rho = \rho_m + 4{\cdot}2\,\omega^2 (r^2 - r_m^2) \times 10^{-10}$ g/cm^3 where r = 5.7 + a/d; a = distance of band from the centre of rotation, d = magnification factor and $\omega^2 = 2.123 \times 10^7$ (Schildkraut

et al 1962).

Determination of mole fraction of 5'-BrdUrd in cellular DNA.

This was calculated using the formulae of Round (1967) and Hackett and Hanawalt (1966).

Determination of the DNA content of cell nuclei by micro-densitometry.

Feulgens reagent was made up as follows: 1.0g basic fushin plus 200ml hot distilled water was filtered. After addition of 20ml 1M HCl and 2.0g potassium metabisulphate the solution was left in the dark until colourless. Discolourisation was ensured by addition of 1.0g activated charcoal mixed for 2 hours and filtered. The resultant reagent was kept in the dark at $0^{o}C$. Sulphite water used to remove non specific staining was freshly made as follows: 10ml of 5% (wt/vol) potassium metabisulphite in distilled water and 10ml of 1M HCl was made up to 200ml with distilled water.

Staining was performed on cells dried onto a microscope slide as follows: The cells were fixed for 15 mins. using ethanol/acetic acid (3:1 v/v) and then washed twice with 95% ethanol, covering the cells for 5 mins. each wash. A small amount of water was added to the slide and 5M HCl added to hydrolyse the sample (20 mins.). The HCl was rinsed away with three changes of distilled water (3 x 5 mins.). The slide was placed in Feulgens stain for 90 mins. in the dark. The stain was rinsed away with three changes of sulphite water and one of distilled water (4 x 5 mins.). Finally the sample was dried using 70% ethanol (5 mins.), 95% ethanol (5 mins.) and drying in air. The sample was visualised under oil immersion with a microdensitometer (Vickers M85 integrating microdensitometer at a wavelength of 560nm). A standard mask was used which just enclosed the largest cell nuclei and the stain quantitated for each nuclei (at least one hundred per sample). Quantitation is determined by absorbance of the sample. The figures presented are in arbitrary units since the instrument was not set up for absolute quantitation.

Preparation of samples for electron microscopy.

The basic method is that of Spurr (1969), using soft resin. 5×10^{7} cells per sample were pelleted (2,000g, 5 mins.) and washed twice with ice cold PBS. The cells were fixed for a minimum of 1 hour in 2% glutaraldehyde in PBS on ice. Excess fixer was washed away with

cold PBS twice and the final cell pellet taken up in 1 ml PBS and spun in a microfuge. The PBS was decanted and 1.0ml of 1.0% osmium tetroxide in PBS added and the cells resuspended. After 1 hour on ice the osmium tetroxide was replaced by 1 ml 1.0% uranyl acetate in distilled water and the sample left for 30 minutes at room temperature. The stain was removed by centrifugation and the sample carefully dried by treatment with increasing concentrations of ethanol in water on ice for 10 minutes each. 20, 40, 60 and 100% ethanol treatments were used. The final ethanol was removed after centrifugation and 250µl of 100% ethanol added. 0.5ml of resin was added and the sample thoroughly resuspended. After 30 minutes at room temperature the tube was topped up with resin and the sample mixed and left for 30 minutes. After centrifugation the sample was re-suspended in neat resin for a further 30 minutes and then this step repeated and the sample left overnight. The sample was then centrifuged and resuspended with 2-3 drops of resin and transferred to embedding capsules, previously air dried at 30-60°C. The capsule was carefully topped up so as not to disturb the pellet (to about 2mm from the top). The resin was the polymerised at 80°C in a preheated oven for at least 24 hours and air cured at room temperature for a further 24 hours.

Thin sections.

Thin sections were cut with a Leitz diamond knife on a Reichert "Om U2" ultramicrotome and transferred to copper grids and dried in air. Further staining was then done with 2% uranyl acetate in distilled water and 0.5% lead citrate in 0.1M NaOH in CO_2-free distilled water as follows: 3 minutes in uranyl acetate, rinse in distilled water, then in 0.01M NaOH, 1 minute in lead citrate, followed by a rinse in 0.01M NaOH and 5 minutes in distilled water. Grids were dried in air before use. A transmission AEI Corinth 275 electron microscope was used to visualise the sections using an accelerating voltage of 60KV. Electron micrographs were taken on 70mm Ilford Line Film N4E50.

Scanning electron microscope.

Samples were prepared as follows below, this being the most suitable method for reducing cell surface serum contamination. Cells were

grown for two days in maintenance medium and transferred to RPMI without serum after washing three times with PBS. After two hours the cells were centrifuges (2,000g for 5 mins.) and resuspended in PBS. Cells were filtered onto Whatman GF/C filters under gravity and cells were fixed in 2.5% glutaraldehyde in cacodylate buffer and critically point dried. Visualisation was in a Phillips 500 scanning electron microscope.

For references to preservation of samples see Anderson (1971) and for critical point drying see Cohen (1974).

Cell size.

Changes in cellular size were recorded using a Model ZBI Coulter counter with a Coulter Channelyser C1000 and XY recorder II. Absolute size was determined using a 12.92u diameter latex bead standard. Samples were diluted 1:4 in "Isopon" (Coulter Electronics Ltd., Harpenden, Herts.) and recorded at an amplification of 32 or 64, current of 1 mA with a 70 um pore. Cell volume was calculated from the following formula:

$$\text{Cell volume } (u^3) = \left(\text{Channel number} + \frac{\text{window width}}{100} \right) \times \text{base channel threshold} \times Tf.$$

Tf. is determined using the latex bead standard assuming a perfect spherical shape.

RESULTS

I. Optimisation of the system for interferon production.

The optimisation of the system requires the variation of two parameters - the amount of inducer and the cell density. The interferon yields from cells induced at differing densities with 150 HAU/ml of Sendai virus are shown in figure 2. The insert shows the actual interferon yields obtained, while the figure itself shows the interferon yield calculated per million cells and plotted arithmetically (instead of logarithmically) to emphasise the narrowness of the optimal region. As can be seen 1×10^6 cells/ml is the optimum and is used hereafter unless otherwise specified. The effect of varying the amount of inducer is shown in Table 1. The optimum amount is about 100 HAU/ml, which was adopted as a standard condition. It can also be seen that very little interferon remains inside the cell after induction and that this does not vary significantly with differing amounts of inducer.

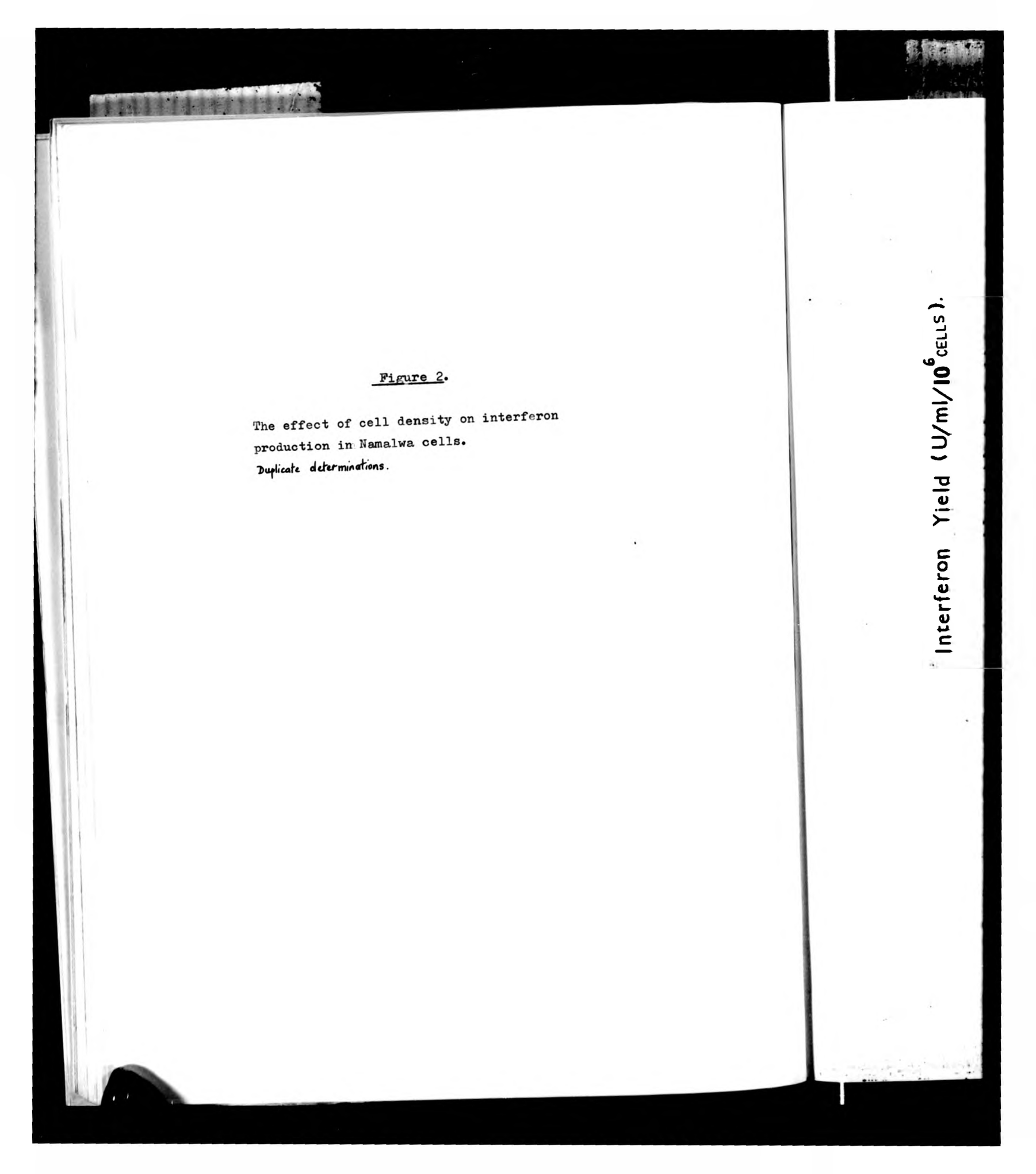
Interferon titres were routinely tested after 24 hours induction. When interferon is titred at earlier times (fig. 8) it can be seen that interferon release is completed by 12 hours.

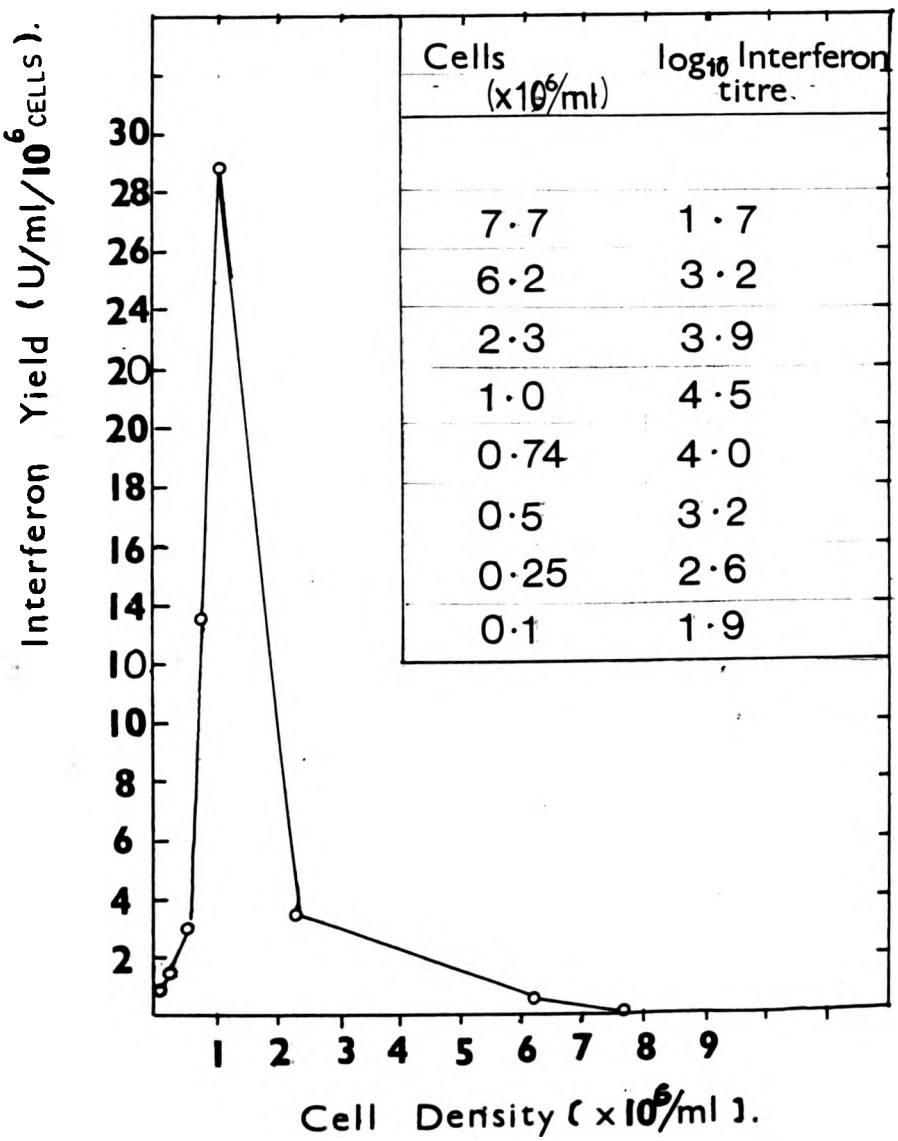
It is not known why Sendai virus is the best reported inducer and because the identity of the inducing molecule itself is not precisely known, finding the answer to this question would be difficult. However a starting point for that kind of study is to investigate the effect of the virus on the producing cell.

The haemagglutination assay for Sendai virus did not reveal any significant quantity of virus even using large infecting doses or high cell densities (Table 2). Also, since the cells were frozen and thawed before assay, there is no large quantity of intracellular virus particles. These titres of virus probably represent virus that had attached to the cells and had not been removed by washing. This contrasts with the replication of Semliki Forest virus in Namalwa cells. Infectious virus as detected by plaque assay is released in large quantities (Table 3). Viral directed RNA, as detected by the incorporation of radioactive uridine into Namalwa cells in the presence of actinomycin D (conditions under which the viral RNA dependant RNA polymerase is active but cellular RNA polymerases

Figure 2.

The effect of cell density on interferon production in Namalwa cells.
Duplicate determinations.





Cells (x10⁶/ml)	log₁₀ Interferon titre.
7·7	1·7
6·2	3·2
2·3	3·9
1·0	4·5
0·74	4·0
0·5	3·2
0·25	2·6
0·1	1·9

Table 1. The optimisation of interferon yields from Namalwa cells with respect to the inducer - Sendai virus.

Sendai virus (HAU/10^6 cells)	Interferon Yield (log_{10} U/ml/10^6 cells)	
	Intracellular*	Extracellular
0.32	< 1	< 1
0.64	< 1	< 1
1.28	ND	2.2
1.60	< 1	2.3
3.20	ND	2.7
6.40	2.2	2.4
12.8	2.0	2.4
19.2	2.2	3.6
51.2	2.1	3.7
96.0	2.4	4.6
192.0	2.5	4.4

ND = Not done.

* Intracellular interferon titred by washing cells with cold PBS (2x), (after removal of extracellular medium by centrifugation), and sonicating the pellet in 2ml of maintainence medium. Titres are uncorrected.

Duplicate determinations.

Table 2. Infection of Namalwa cells with Sendai virus.

Sendai virus HA/10^6 cells	Virus titre (HA/ml) (24 hours post infection)
3.2	<8
6.4	<8
12.8	<8
32	8
96	8
320	16
960	32

Cell density* $x10^{-6}$ (cells/ml)	Virus titre (HA/ml) (24 hours post infection)
0.4	<8
0.6	<8
0.8	<8
1.0	<8
2.0	8
4.0	8
6.0	8
8.0	16
10.0	16

* Namalwa cells infected with 100 HA/ml Sendai virus.

Duplicate determinations.

Table 3. **Cumulative yields of SFV released from infected Namalwa cells.**

Time post-infection (hours)	Infectious virus (pfu/ml)
2	1.5×10^3
3	1.5×10^3
4	1.6×10^3
5	3.6×10^3
6	1×10^4
7	2×10^4
8	2.3×10^4

Yields from Namalwa cells infected with ca. 50 pfu/ml SFV (from stock 1.2×10^8 pfu/ml), the cell density being 1×10^6/ml.

Two experiments with duplicate determinations.

are largely inhibited), is also accumulated in large quantities (fig.3). This shows that in spite of the report that Namalwa cells produce small quantities of autogenous interferon (Adams et al 1975, Tovey et al 1977), the Namalwa cells are not in a self-induced 'anti-viral' state, since infectious SFV is produced. Thus Sendai virus replication is unlikely to be inhibited by this interferon. Sendai virus replication in Namalwa cells thus appears to be abortive and the block must occur before maturation of the virus. If double-stranded RNA is the inducer, then the virus must at least be able to infect the cells and replicate its RNA.

The effect of Sendai virus on cellular macromolecular synthesis was investigated by studying the incorporation of radiolabelled precursors of protein, RNA and DNA synthesis and by resolution of individual proteins on two dimensional polyacrylamide gels.

There appears to be a slight but reproducible reduction in the incorporation of radiolabelled precursors into TCA precipitable material (fig. 4 and Table 4) but no large change is seen, as may occur if the Sendai virus is replicating to a significant extent. The incorporations are presented as the ratio of incorporation between infected and uninfected cells since the actual incorporation varies between experiments, while this ratio remains similar. The depression is at the limit of determination of the method but consistent. Table 4 clearly shows that thymidine incorporation is reduced by around 30% while uridine and methionine incorporation are depressed by 10% or so. The pool sizes of the radiolabels as measured qualitatively by TCA soluble uptake into Namalwa cells shows a similar fluctuation for uridine and methionine to the TCA precipitable determinations. The thymidine pool sizes determined this way show that the ratio between infected and uninfected cells is about unity. Thus the depression observed for thymidine incorporation into DNA is independant of changes in the thymidine pool. Whether the depressions in the pool size of the two other precursors represents the cause or effect of the depression observed for protein and RNA synthesis was not determined.

Analysis of the proteins synthesized in uninfected and infected Namalwa cells was determined by two dimensional electrophoresis (fig. 6). The proteins produced are essentially similar, with a few

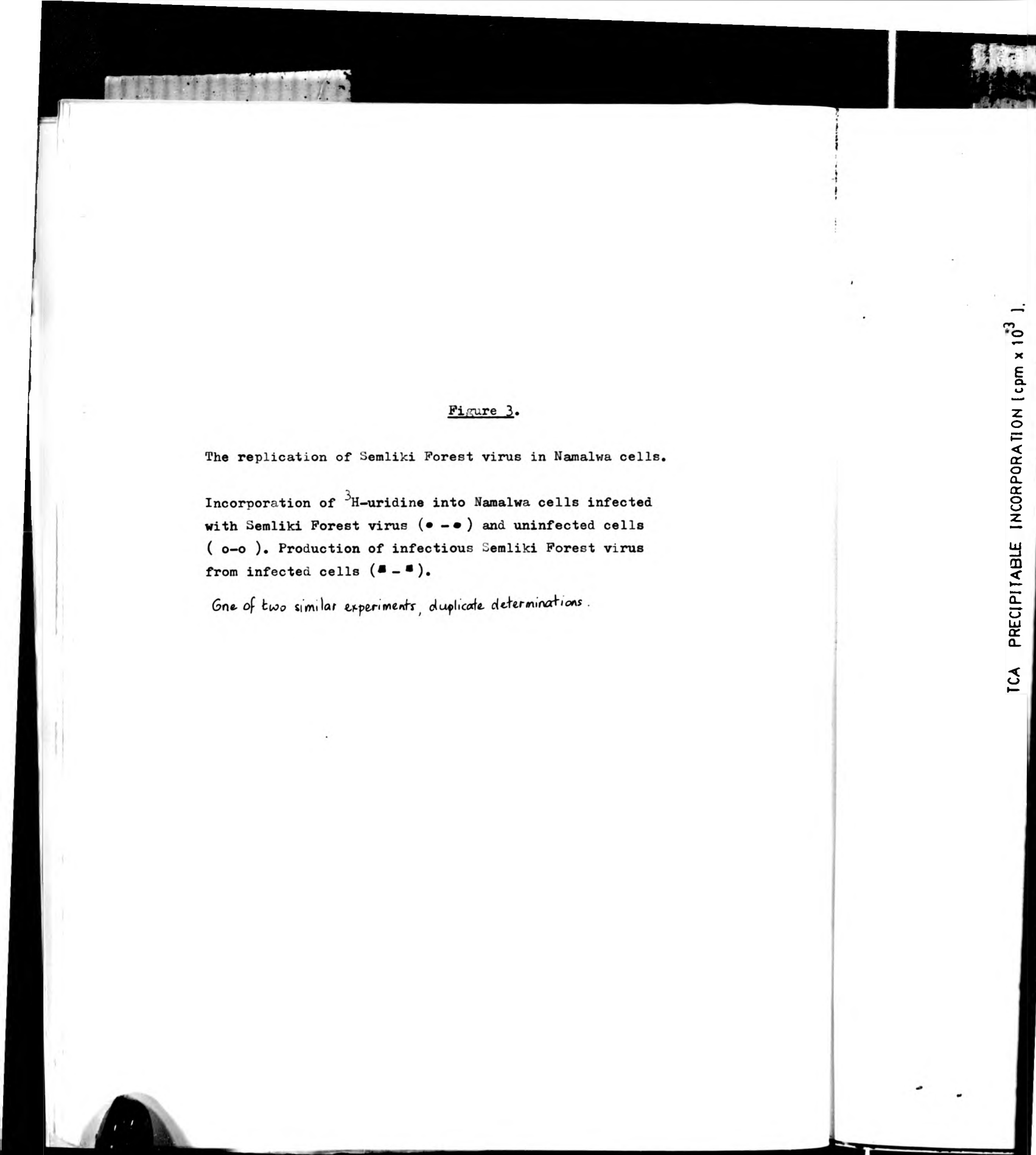


Figure 3.

The replication of Semliki Forest virus in Namalwa cells.

Incorporation of ^{3}H-uridine into Namalwa cells infected with Semliki Forest virus (• – •) and uninfected cells (o–o). Production of infectious Semliki Forest virus from infected cells (■ – ■).

One of two similar experiments, duplicate determinations.

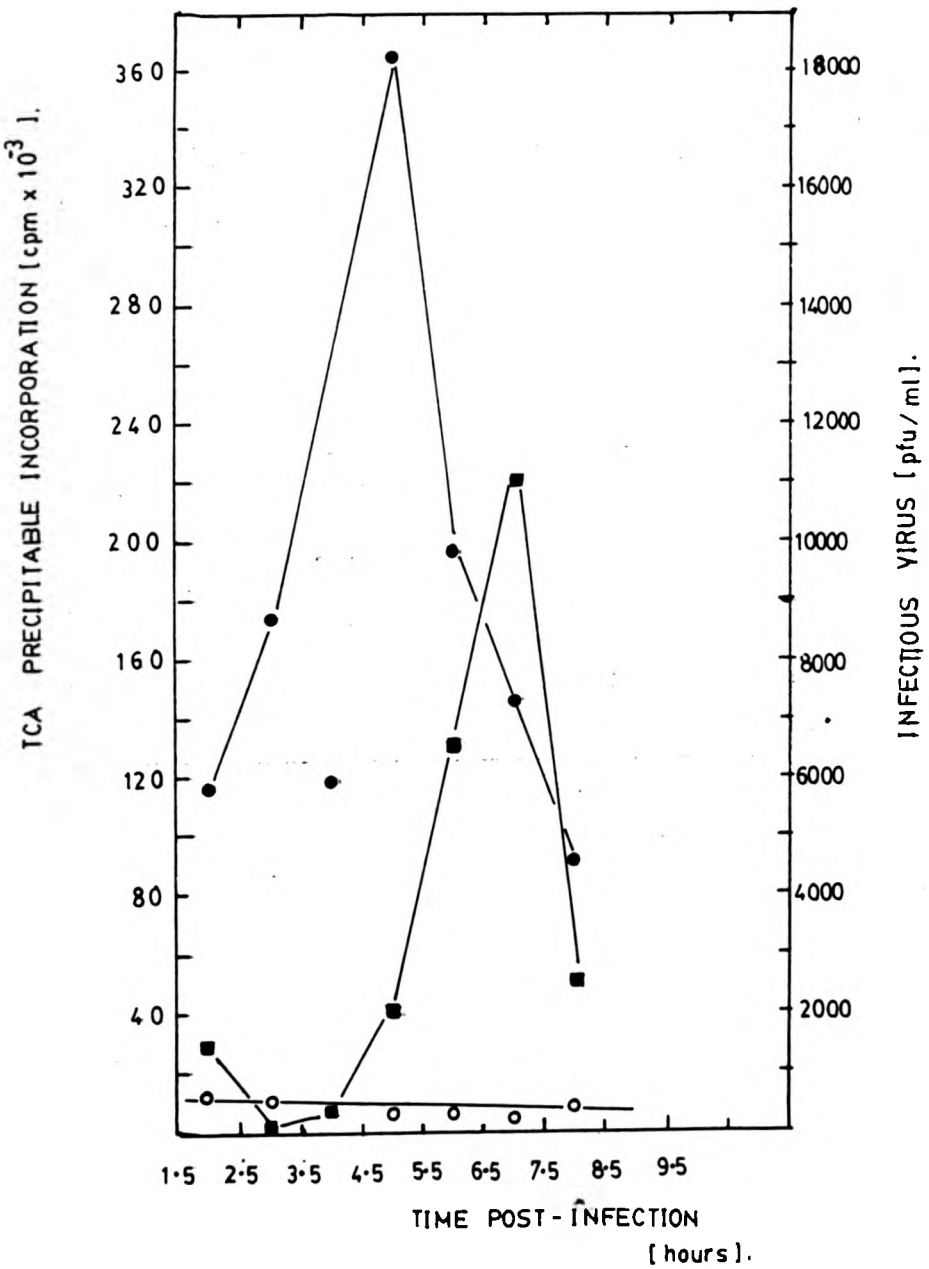

TCA PRECIPITABLE INCORPORATION [cpm x 10^{-3}].
360
320
280
240
200
160
120
80
40
18000
16000
14000
12000
10000
8000
6000
4000
2000
INFECTIOUS VIRUS [pfu/ml].
1·5 2·5 3·5 4·5 5·5 6·5 7·5 8·5 9·5
TIME POST-INFECTION
[hours].

Figure 4.

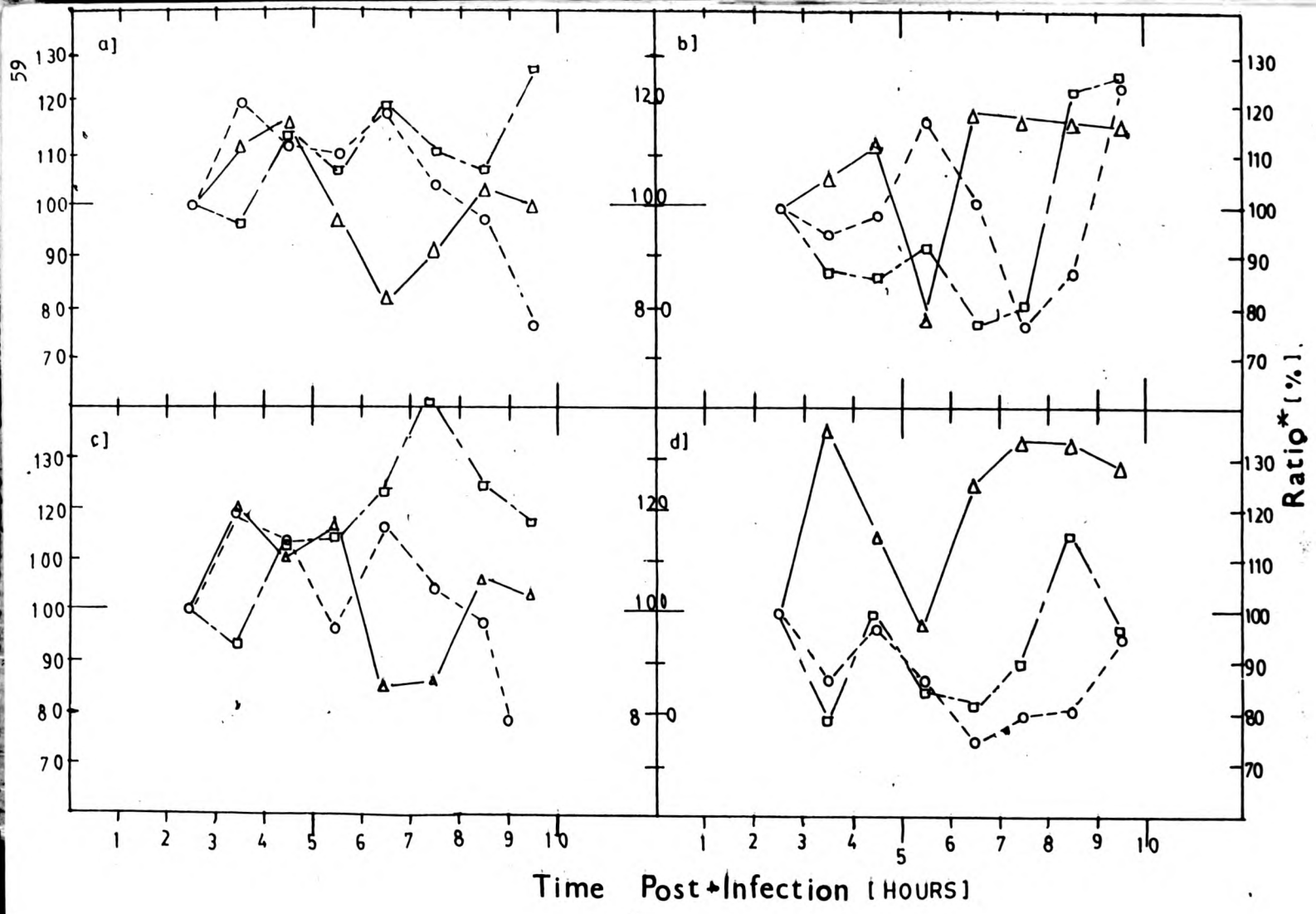
The effect of Sendai on macromolecular synthesis in Namalwa cells.

Incorporation of labelled uridine (Δ -Δ), methionine (□ -□) and thymidine (O–O) into Namalwa cells.

a) uninfected Namalwa cells -TCA precipitable incorporation.
b) uninfected Namalwa cells -TCA soluble incorporation.
c) Sendai infected Namalwa cells -TCA precipitable incorporation.
d) Sendai infected Namalwa cells - TCA soluble incorporation.

* Incorporation is determined as the percentage of the counts per minute recorded compared to the incorporation (of the given isotope) determined as the first point and defined as 100%.

Averages of two determinations for each point and three experiments. Table 4 shows same data in numerical form.



...ion.
...ation.
...counts
...he
...d
...experiments.

Table 4a. Incorporation of uridine, methionine and thymidine into TCA precipitates of Namalwa cells.

Uridine	Time post-infection (hours)							
	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
Infected cells	100	120	110	116	85	86	106	103
Uninfected cells	100	111	116	97	81	91	103	100
Methionine								
	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
Infected cells	100	93	112	114	123	141	124	117
Uninfected cells	100	96	114	107	119	111	107	127
Thymidine								
	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
Infected cells	100	119	113	98	116	100	94	88
Uninfected cells	100	120	112	110	118	104	97	78

Table 4b. Incorporation of labelled uridine, methionine and thymidine into Namalwa cells - TCA soluble determinations.

Uridine	Time post-infection (hours)							
	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
Infected cells	100	136	115	99	126	134	132	129
Uninfected cells	I00	106	113	77	119	117	117	116

Methionine								
	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
Infected cells	100	87	97	87	75	80	81	95
Uninfected cells	100	94	98	117	101	77	87	124

Thymidine								
	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
Infected cells	100	79	100	85	82	90	118	97
Uninfected cells	100	87	86	92	78	81	123	126

Figure 5.

Ratio of the incorporation of labelled uridine, methionine and thymidine into infected and uninfected Namalwa cells.

Incorporation of labelled uridine (Δ-Δ), methionine (□-□) and thymidine (○-○) into Namalwa cells.
a)TCA precipitable incorporation.
b)TCA soluble incorporation.

* Incorporation is determined as the ratio between the count per minute recorded for Sendai infected Namalwa cells and uninfected cells.

Means of 3 experiments with duplicate determinations.

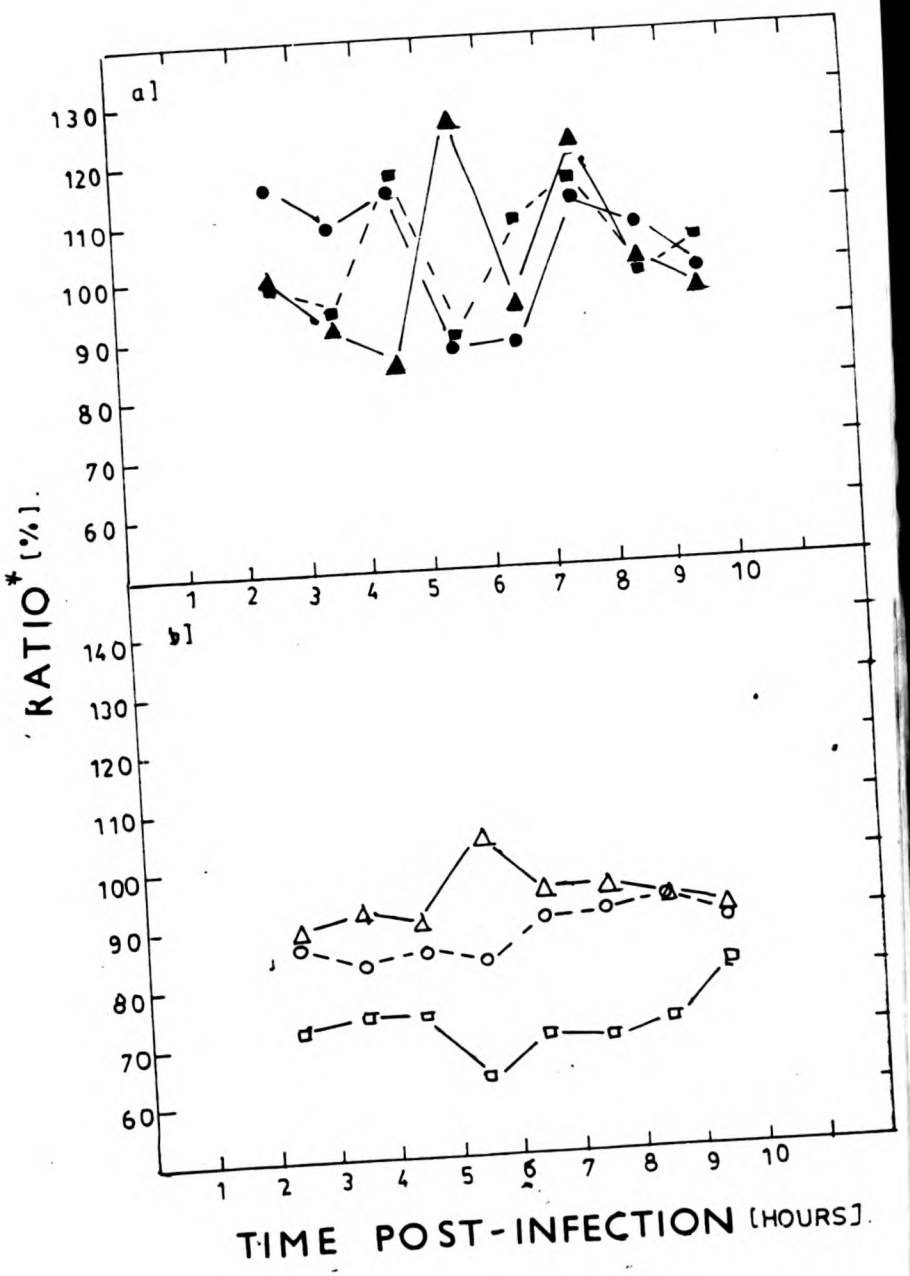

a]
b]
130
120
110
100
90
80
70
60
140
1 2 3 4 5 6 7 8 9 10
RATIO* [%].
TIME POST-INFECTION [HOURS].

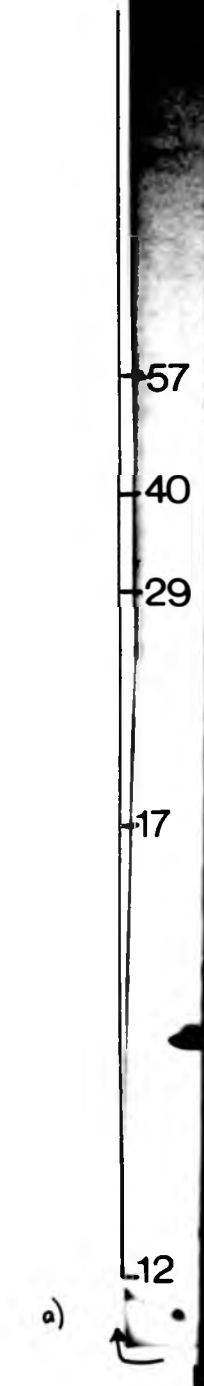
Figure 6.

Two dimensional gel electrophoresis of infected and uninfected cells.

a) Namalwa cells infected with Sendai virus and labelled with ^{35}S-methionine.

b) Uninfected cells.

These gels were prepared by the method of O'Farrel (see Methods for details).



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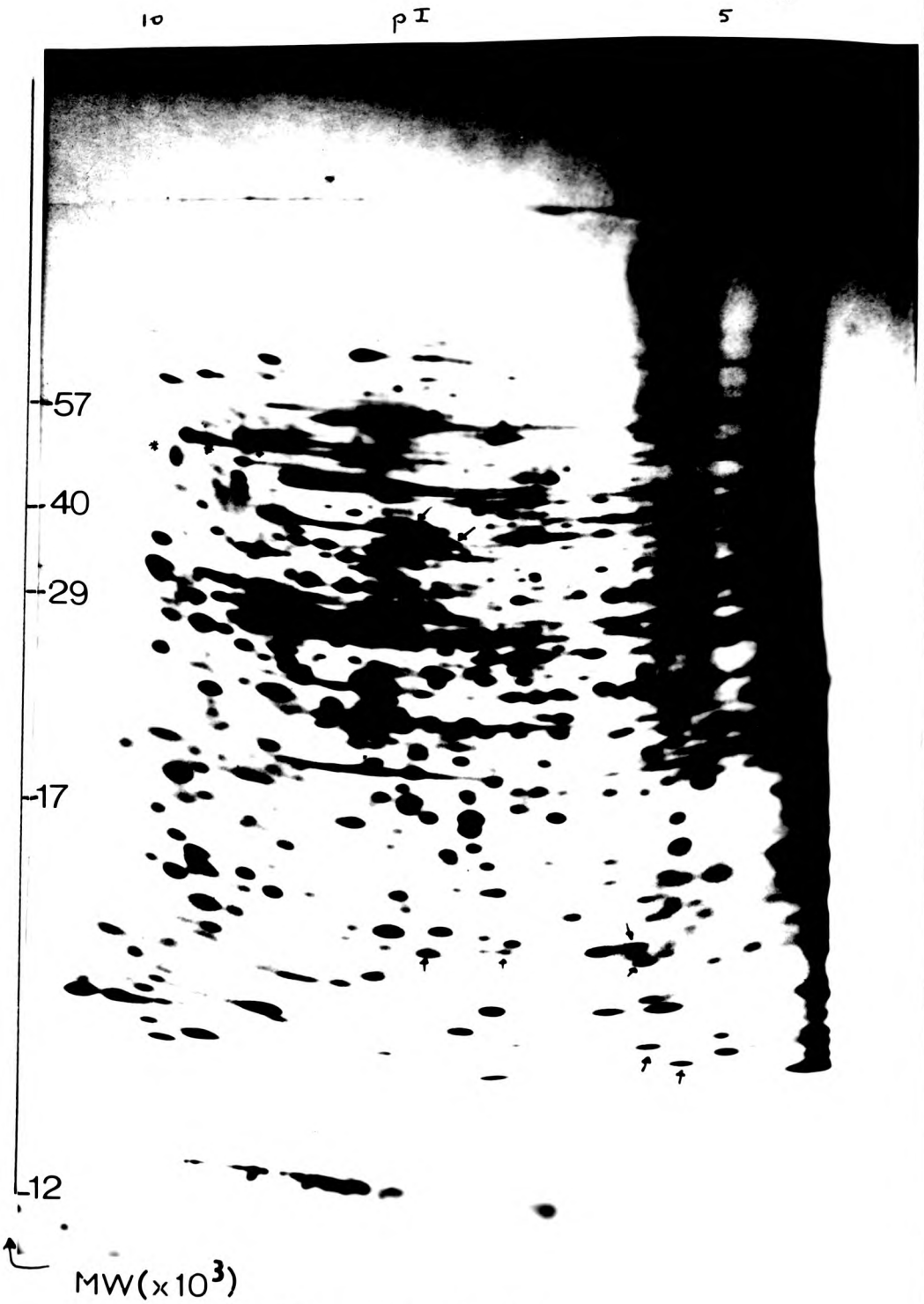
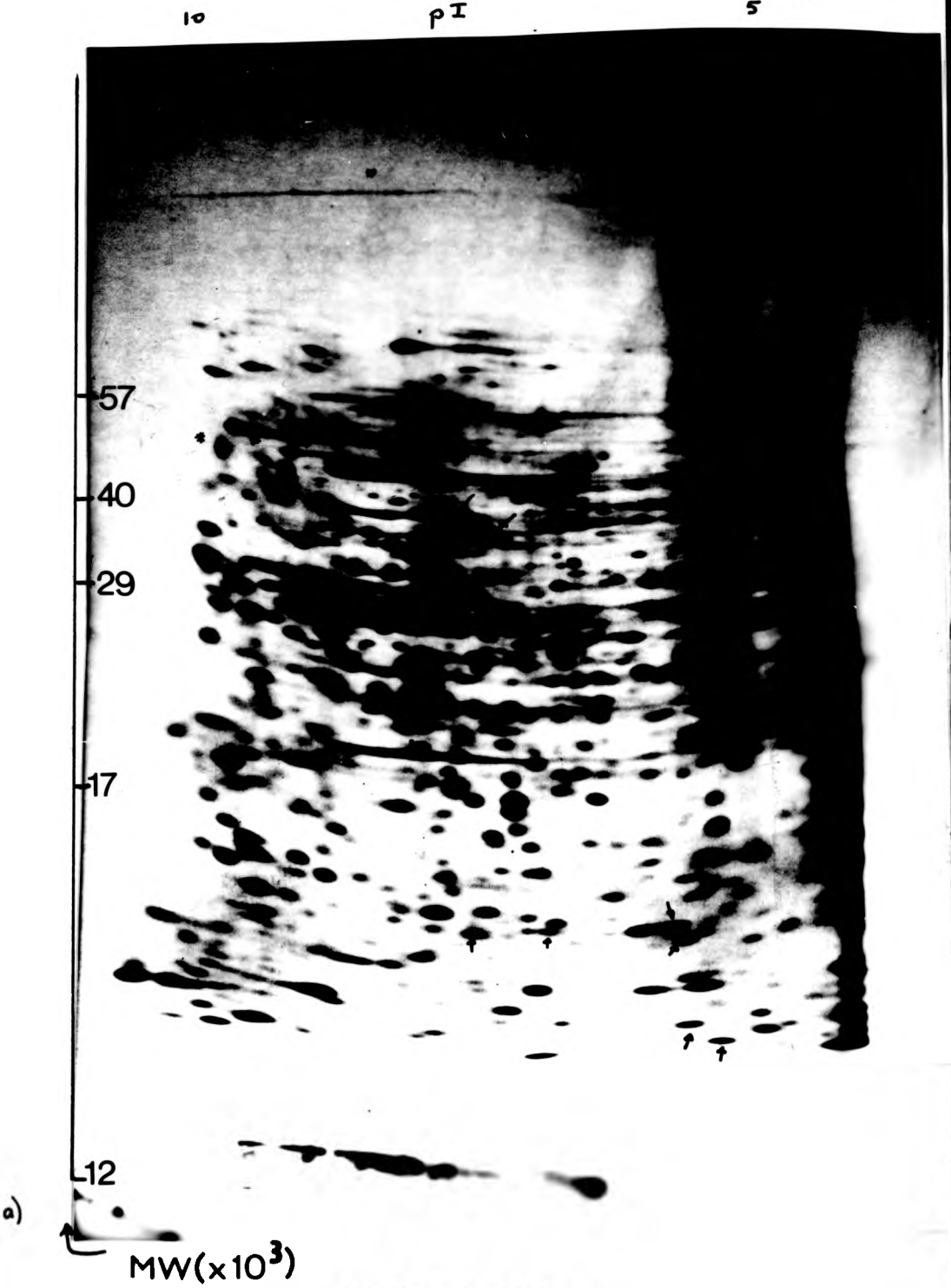


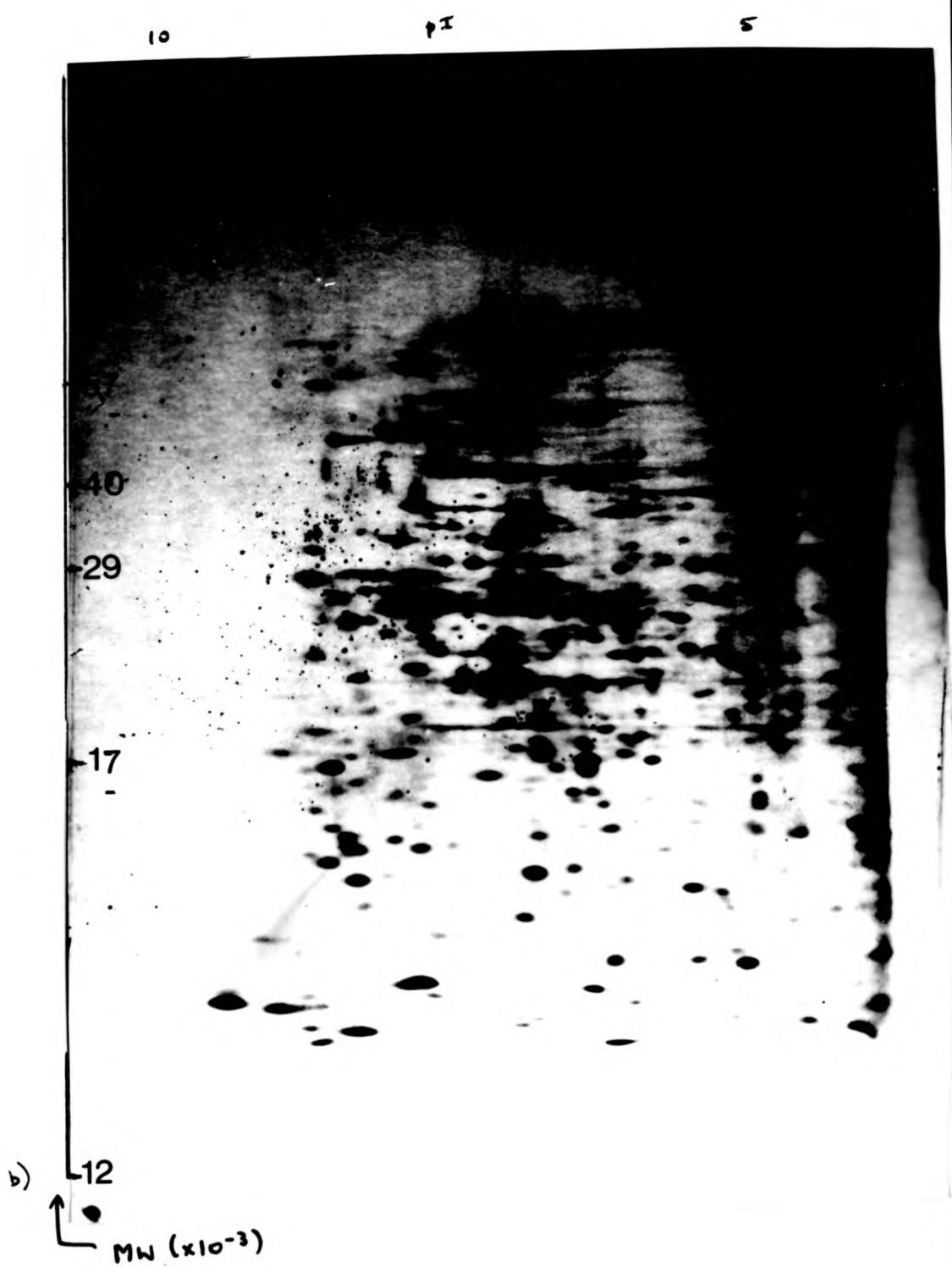
NB. ARROWS SHOWS DIFFERENCES FROM GEL b)

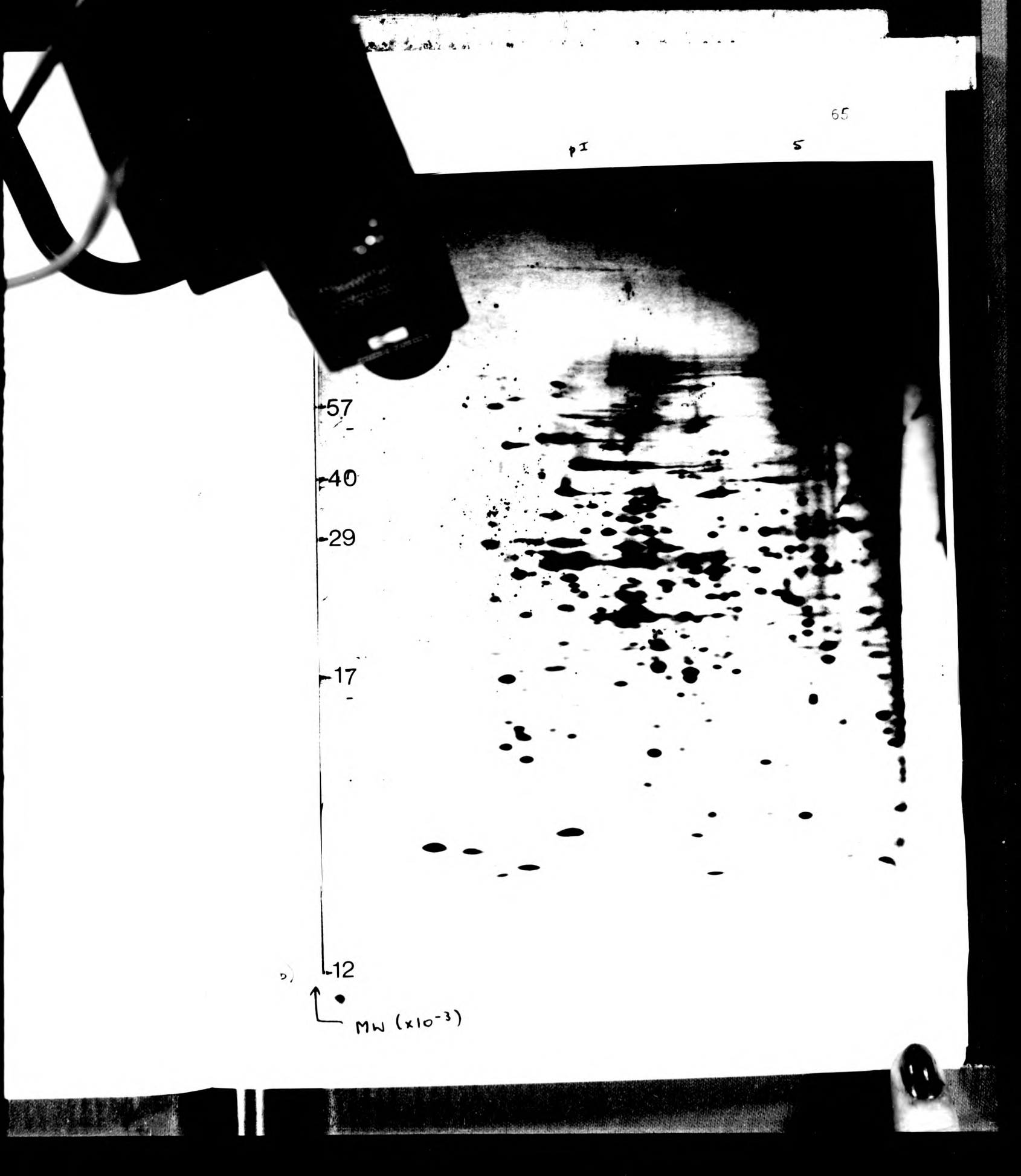
belled

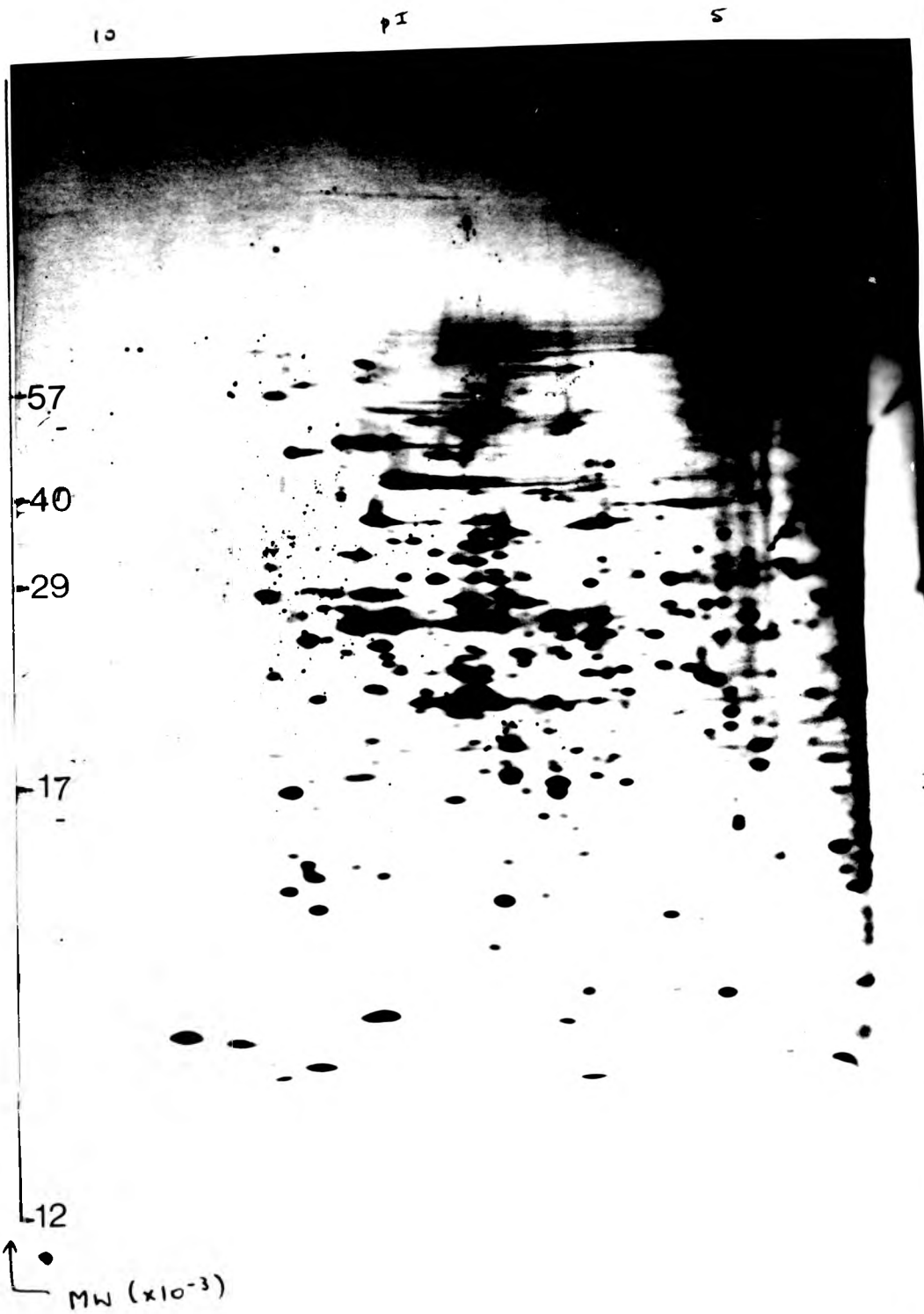
see Methods



NB. ARROWS SHOWS DIFFERENCES FROM GEL a)


10
pI
5
40
29
17
12
b)
MW (x10⁻³)


pI
5
57
40
29
17
12
MW (x10⁻³)


10
pI
5
57
40
29
17
12
MW (x10⁻³)

additional proteins produced in infected cells (arrows). Certain of these additional proteins have the same molecular weight but several isoelectric-focussing points (pI's). This may be due to the presence of varying amounts of carbohydrate on identical protein. Only a few gels were run so it is possible that these additional spots are not reproducible. Certainly no major new spots can be seen in infected cells suggesting that Sendai specified proteins are not made to any significant extent in Namalwa cells.

Thus it seems that Sendai virus has very little effect on Namalwa cells and does not exhibit host cell cut off. The proteins that are possibly viral specified are not present in high concentrations as judged from their density relative to other cellular proteins. This supports the idea that Sendai infection of Namalwa cells is an abortive infection. One reason why Sendai virus is a good inducer may result from the fact that interferon production is not interfered with by any anticellular effect of the viral replicative cycle over the period of interferon production i.e. 12 hours.

II. Cloning of Namalwa cells.

Namalwa cells make very variable yields of interferon in response to Sendai virus. The variation can be due to the presence of too low a quantity of inducer (presumably not all the cells are infected) or due to the effects of cell density as first reported by Strander, Mogenson and Cantell (1975). The reason for the latter effect is not known. However under standard conditions variation still occurs. This variation has implications in determining the significance of any given result and may also be important if it reflects the quantity of mRNA for interferon present in the cells for both the oocyte assay (see above) and purification of IFmRNA and for the preparation of a radioimmuno-assay to replace the biological assay for interferon activity.

A possible explanation for the variation is that not all the cells in a culture make interferon or that sub-populations of cells exist that make different amounts of interferon. There are two ways to investigate this problem. One is to measure the interferon yields of individual cells (Rodgers and Merrigan 1974) which limits the number of cells one can look at, or use the single cell assay technique of Kronenburg (1976). Alternatively the cells can be cloned from individual cells. Since Namalwa are Burkitt lymphoma cells they have a high plating efficiency in agar (Nilsson and Ponten 1975). Thus the agar-pearl technique of Hinamura and Grace (1968) can be used. Other investigators have sucessfully used this method with other lymphoblastoid cell lines, though chiefly to determine Epstein-Barr virus genome contents per cell (Zajac and Kohn 1970, Maurer, Inamur and Wilbert 1970, Zajac, Henle and Henle 1969).

Other methods are available for cells that will not grow in agar such as the use of feeder layers and liquid culture (Fresen et al 1977, and Miller, Stitt and Miller 1970, Povey et al 1973).

Individual colonies can be picked and grown up to cultures large enough to be induced for interferon. There are problems with this technique as the karyotype of the clones does not appear

to be stable and a heterogeneous population occurs relatively rapidly. Several determinations are thus required to determine stability and variation.

Results.

Clones of Namalwa cells were obtained over a period of three months, their establishment being slow and difficult. Ten clones were selected and tested for their interferon production (Table 5). Subsequently the clones could not be maintained and recovery of the cells from liquid nitrogen was very poor which inhibited their reestablishment. The reason for this behaviour is not understood.

Clumping of the cells varied between lines (compare fig.7a with fig.7b) although this is dependant to some extent on cell density (compare fig.7b with fig.7c). The growth rates (fig.8) were significantly different but interferon production showed variation between clones that was only at the limit of error in the assay determinations (Table 5). Variation between passages is also as high. It seems unlikely that the variation that may be present between clones could account for the variations seen in the normal parental culture. It is possible that the selection favours fast growers and that such cells all produce interferon.

A subsequent cloning using 10 x RPMI 1640 medium in place of 10 x GMEM medium led to the rapid establishment of clones which could be maintained for long periods with good viabilty and although recovery from liquid nitrogen was poor, lines could be reestablished. This set of 22 clones did not contain any clone making more than 2.0 $10g_{10}$ U IF/ml (lower titres were not looked at).

The contrast between these two sets of clones cannot be explained though culture conditions varied between the two - the former being buffered with bicarbonate and CO_2 and the latter with hepes.

It is possible that the stage in the cell cycle is important for interferon production. Cell synchronisation would answer this question although only partial synchronisation of lymphoblastoid cell lines can be achieved (Zielke and Littlefield 1972).

Table 5 — Induction of Namalwa clones for interferon.

Interferon Yield (log_{10}U/ml/10^6 cells).

Clone	Passage 6	7	8	9	10	11	12	13	14	15	16
1	<2·2	2·7	2·8	2·0							
2			3·1	3·7	3·6	<1·8					
3								<2·2	3·2	3·6	1·8
4	2·3	2·4	2·9	1·7							
5							2·2	2·7	<1·8		
6	<2·2	2·8	3·2	2·5							
7				2·8	3·2	3·8	<1·8				
8			3·1								
9				<2·2	2·7	3·25	2·8				
10				<2·3	2·4	2·8	<1·8				

Interferon Yield† (log_{10}U/ml/10^6 cells).

Clone	Passage 7	8	9	10	11	12	13	14
1	3·3							
2		3·2	3·7					
3								3·7
4	3·5							
5						4·2		
6	3·7							
7			3·7	4·2				
8			3·6					
9				3·3				
10				3·7				

† Cells pretreated with 25 ug/ml BrdUrd for 48 hours.

a)

b)

Figure 7.

Namalwa clones - morphology.

Namalwa cells after 24 hours growth in 25cm^3 tissue culture flasks. Magnification x 200.

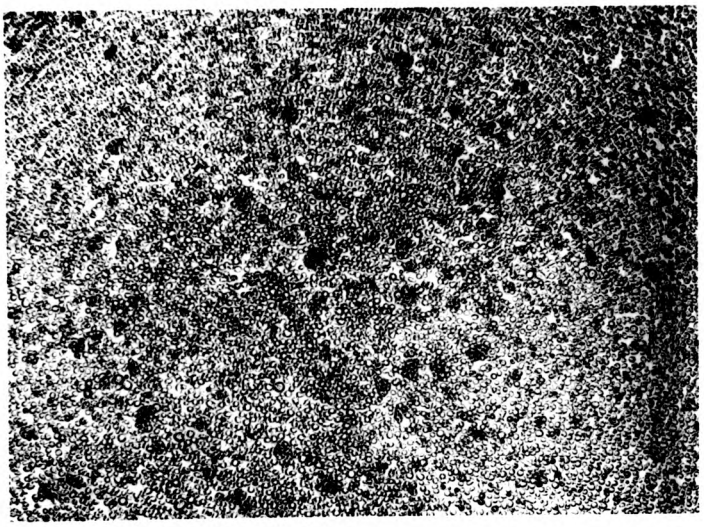
a) Parent cell line.

b, c and d) Three clones derived from the parent line.

culture

ine.

a)



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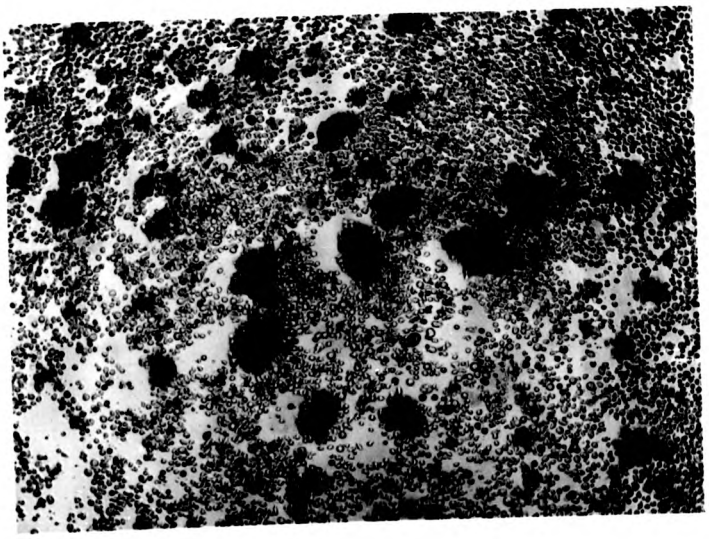

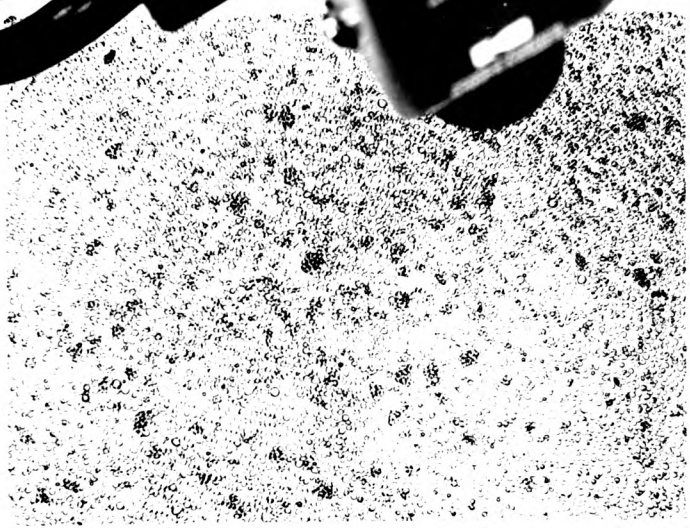

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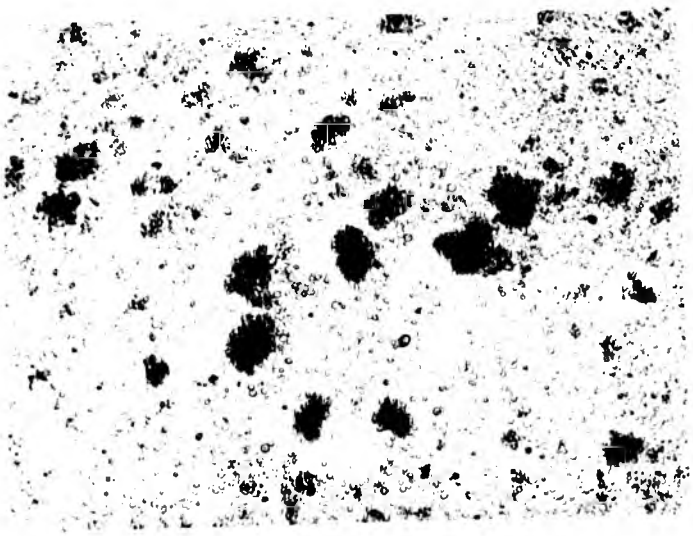

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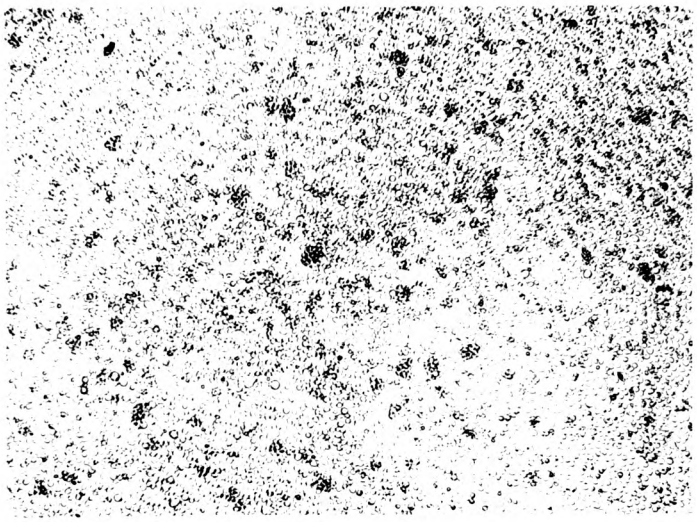

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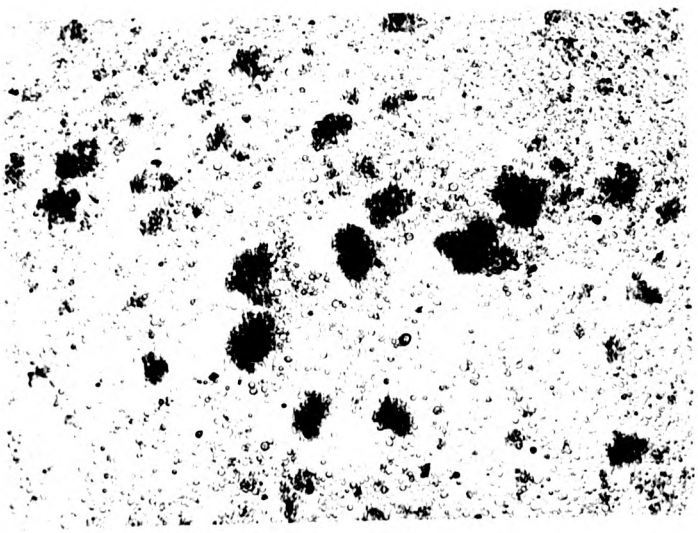

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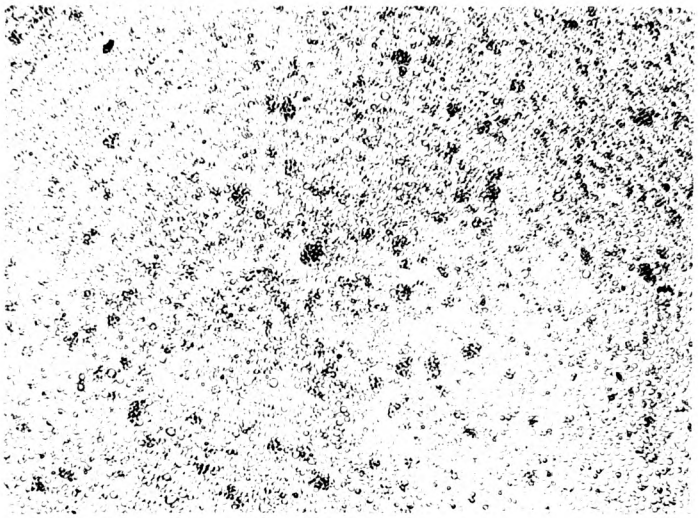

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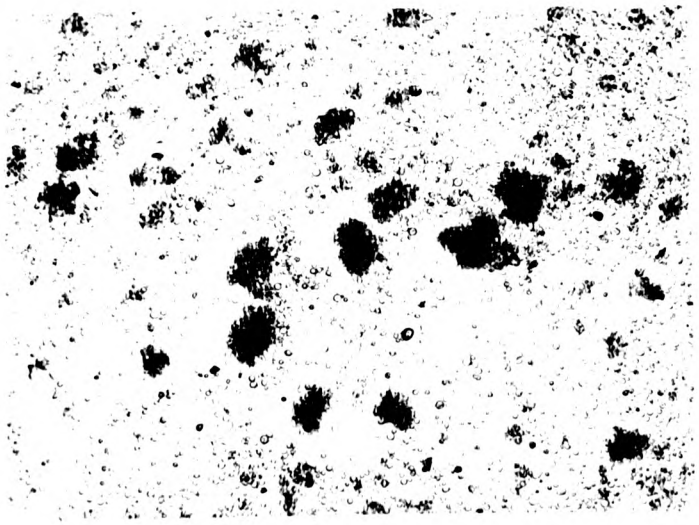

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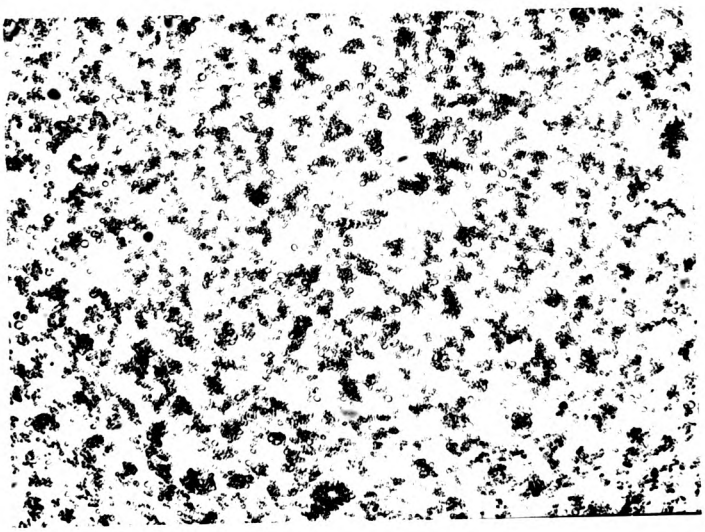

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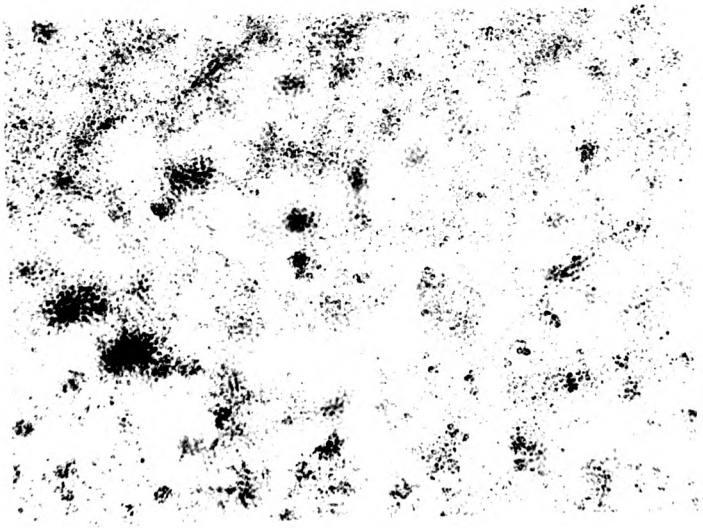

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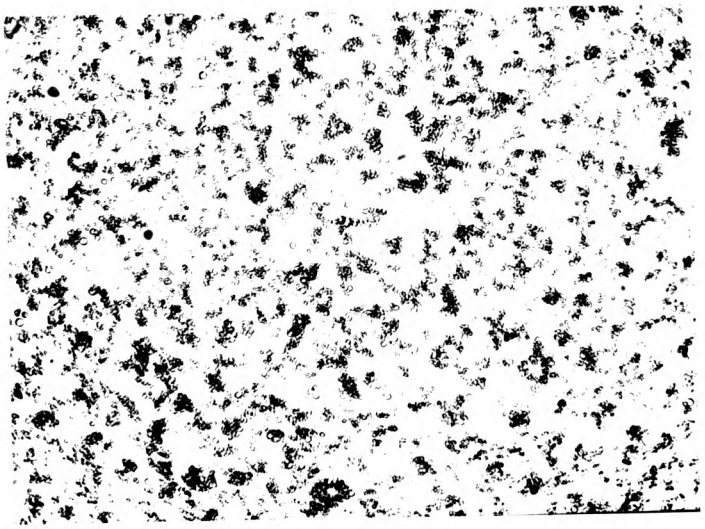

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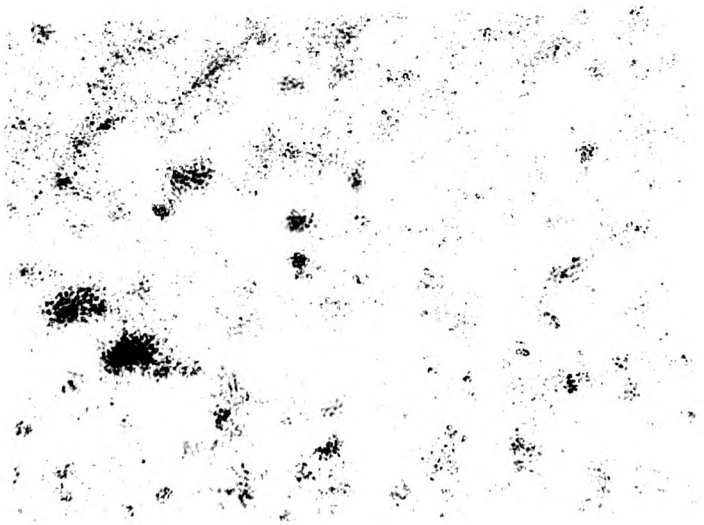


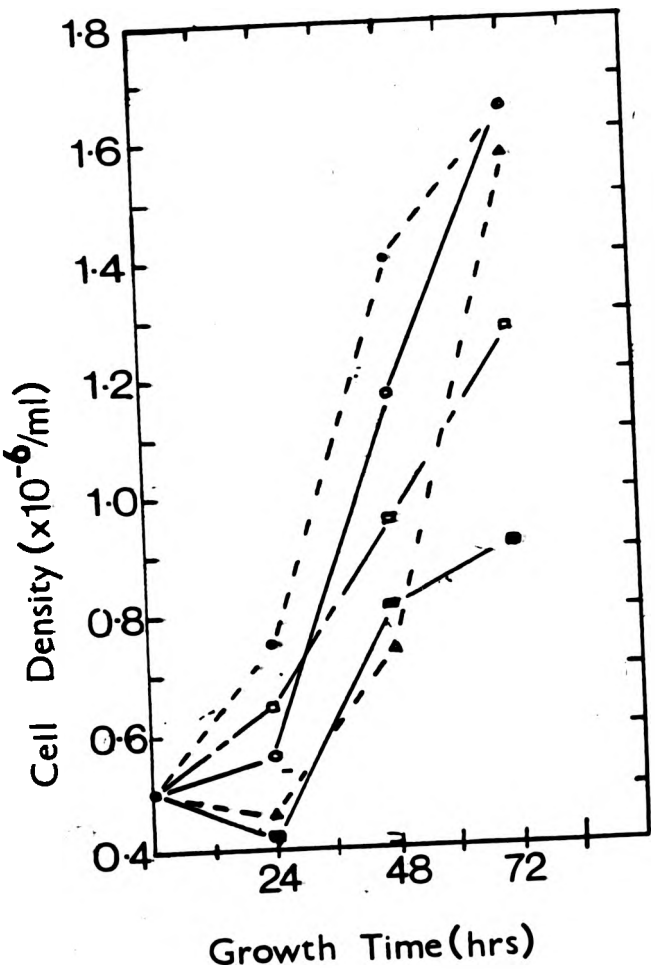
Figure 8.

Namalwa cells - growth rates.

Growth of clones from 0.5 x 10^6/ml over three days.

(o–o) Parent cell line.
(●–●) Clone 1.
(□ – □) Clone 2.
(■ – ■) Clone 3.
(▲ – ▲) Clone 4.

Mean of two experiments with duplicate determinations.


1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
Cell Density (x10⁻⁶/ml)
24
48
72
Growth Time (hrs)

Double thymidine or other induced blocks can cause perturbations in cellular metabolism thus complicating the interpretation of any results. Thymidine for instance is well known to cause toxicity and even mutagenesis in mammalian cells probably as a result of changes caused by thymidine in the intracellular pools of the other deoxynucleotide triphophates (Bradley and Sharkey 1978, Saffhill and Abbot 1979).

III. 5'-bromodeoxyuridine. Its effects and mode of action.

5'-bromodeoxyuridine is an analogue of thymidine and is incorporated by most cells into cellular DNA in place of thymidine, when added exogenously. It is not known whether 5'-bromodeoxyuridine must be incorporated into DNA before its effects are manifest. Certainly in neuroblastoma cells it seems that a non-DNA linked mechanism is indicated (Schubert and Jacob 1970).

5'-bromodeoxyuridine has a specific effect in inhibiting particular stages of differentiation in several cell systems (for review see Rutter et al 1973). The effect involves both the inhibition of growth (to varying degrees) and the specific inhibition of the production of specialised proteins made in differentiating cells (Bischoff and Holtzer 1970, Coleman et al 1970, Lasher and Kahn 1970, Stellwagen and Tomkins 1970, 1971, Weintraub et al 1972, Davidson and Bick 1973, Walther et al 1974, O'Brien and Stellwagen 1977 and Dudits et al 1979). The effect is not simply due to a depression of RNA or protein synthesis, since many of these effects occur at concentrations less than that required to significantly inhibit such biosynthesis (Stellwagen and Tomkins 1971, Weintraub et al 1972, Pictet et al 1974 and Dudits et al 1979). Furthermore, the effect on tyrosine aminotransferase is rapid and independent of the normal steroid inducers. There is also a differential rate of inhibition of enzyme activities and some enzymes are not even affected (Stellwagen and Tomkins 1971 and O'Brien and Stellwagen 1977). The time of treatment with 5'-bromodeoxyuridine is very important. There is no effect on the development of haemoglobin synthesis in erythroblasts treated with 5'-bromodeoxyuridine after haemoglobin synthesis has started, whereas it is completely inhibited if 5'-bromodeoxyuridine is given prior to differentiation. A 3-fold higher concentration given after differentiation still has no effect (Wientraub et al 1972).

Paradoxically, 5'-bromodeoxyuridine can also increase the production of certain enzymes and proteins (Koyama and Ono 1971, 1972, Bick and Soffer 1976, Biowas et al 1977 and Tovey et al 1977). Such increases can occur in the same cells where 5'-bromodeoxyuridine

inhibits other enzymes and does not affect others at all (O'Brien and Stellwagen 1977).

All the effects are completely reversible, indicating that cellular damage is slight at the concentrations used. It seems unlikely that the mutagenicity of 5'-bromodeoxyuridine plays a significant role in its specific effects. The length of time required for induction or inhibition is very short and the cells can grow for many population doublings with their DNA totally substituted with 5'-bromodeoxyuridine for thymidine without any great effect (Davidson and Bick 1974, Bick and Soffer 1976 and Kaufman and Davidson 1977).

Other effects of 5'-bromodeoxyuridine are the reduction of the tumourigenicity and acquisition of contact inhibition (Siliga and Bruce 1970) and effects on the cell surface (Kilton and Mach 1977). Large morphological changes, referred to as a general 'transformation', can also occur (Davidson and Horn 1974).

5'-bromodeoxyuridine is also known to induce 'C'-type and other latent viruses (Aaronson et al 1971, Hampar et al 1971, Klement et al 1971, Lowy et al 1971, Sugawara et al 1972, Stewart et al 1972a, 1972b and Teich et al 1973). The time course of this effect is longer than the others (Hampar et al 1971, 1973, Lowy et al 1971 and Sugawara et al 1972), and seems to require incorporation of 5'-bromodeoxyuridine into DNA (Teich et al 1973) and in the case of Epstein-Barr virus, the activation is S-phase specific (Hampar et al 1973). The induction occurs in every cell in Balb C mouse embroyo 3T3 cells as shown by cloning the cell population from single cells (Aaronson et al 1971). The induction of the defective murine sarcoma virus (MSV) by 5'-bromodeoxyuridine occurs in the absence of helper virus murine leukaemia virus (MLV) and without any detectable MLV-specific antigen being formed (Klement et al 1971), and therefore one cannot explain the high numbers of cells that are induced to form virus by the mutagenic action of 5'-bromodeoxyuridine. The chemically induced virus is antigenically different from MSV but the physical characteristics of the virus are similar to the MLV/MSV complex. The probable explanation of the effect of 5'-bromodeoxyuridine in the rat cells used is that a rat C -type virus is induced by the treatment and that this C-type virus performs the

helper virus functions required for the defective MSV to replicate. It is also possible, however, that 5'-bromodeoxyuridine treatment causes a mutation in the MSV genome or causes elimination of a specific repressor, such that the MSV could replicate. Thus it is possible that 5'-bromodeoxyuridine disrupts the normal regulatory mechanisms that suppress virus formation in latently infected cells. It has been shown that the lac repressor binds with a 10-fold higher affinity to 5'-bromodeoxyuridine-substituted lac operon DNA than unsubstituted lac operon DNA, whether or not the inducer of the lac gene, isopropylthiogalactoside, is present (Lin and Riggs 1972). They suggested that the consequent decrease in the transcription of the lac gene resulted not just from a reduction in template activity, but by increasing the affinity of chromosomal proteins which are regulated. Thus constituitive enzymes would be less sensitive to inhibition by 5'-bromodeoxyuridine and this seems to be the case (Stellwagen and Tomkins 1971).

It may be that other modulators of gene expression have a similar mechanism. Examples of specific gene expression induced by seemingly non specific treatments are heat shock and treatment of certain cells with amino acid analogues. Specific patterns of new gene expression are seen following heat shock of polytene chromosomes in Drosophila cells (Lewis et al 1975). Treatment of cells with amino acid analogues or mild heat shock induces the synthesis of three proteins, which then dominate the bio-synthetic capacity of the cells (Kelly and Schleisenger 1979).

Several studies have shown that 5'-bromodeoxyuridine substitution of DNA alters the template activity of chromatin (Hill et al 1973, Kotzin and Baker 1972, Matsumoto et al 1975 and Lykkesfeldt and Anderson 1977), both in vitro and in vivo. Studies on chromatin in vitro have shown that the thermal stability of DNA is higher when the DNA is substituted with 5'-bromodeoxyuridine. A condensation of the chromatin also occurs (Lapeyre and Bekhor 1974). This may be accounted for by an increased affinity of chromosomal proteins for the substituted DNA (David, Gordon and Rutter 1974). There is evidence for this suggestion, both for histones and non-histone

proteins (Gordon et al 1976 and Schwartz 1977), though the latter show a much greater selectivity towards substituted DNA than the histones. This is interpreted as a specific affinity of substituted DNA for certain chromosomal proteins (Gordon et al 1976).

Mutagenesis as a mode of action for 5'-bromodeoxyuridine has not however been ruled out entirely. The molecules of glucose-6-phosphate dehydrogenase (G6PdH) from 5'-bromodeoxyuridine treated hamster cells have an altered heat lability and a different affinity for the co-factor NADP compared to native enzyme. This altered population of molecules increases with increasing substitution of 5'-bromodeoxyuridine into cellular DNA over 300 population doublings (Bick and Soffer 1976). The enzyme normally shows two peaks of activity, the second of which increases relative to the first peak after 5'-bromodeoxyuridine treatment. This suggests that this fraction is the heat labile fraction. It is possible that there could be two gene loci for G6PdH. Then the alteration in enzyme activity could be accounted for a change in transcription. The interpretation placed upon the results by the authors was that 5'-bromodeoxyuridine causes base sequence changes in the DNA and thus alters the primary sequence of the protein, which consequently has altered the physical properties of the protein.

5'-bromodeoxyuridine and deoxythymidine both cause mutagenesis. They also inhibit the enzyme ribonucleotide reductase at certain concentrations (Reichard, Canellakis and Canellakis 1961 and Meuth and Green 1974). This enzyme catalyses the reduction of nucleotide diphosphates and deoxynucleotides. The action of deoxythymidine and 5'-bromodeoxyuridine preferentially affects the reduction of uridine and cytidine diphosphates. (Moore and Hurlbert 1966 and Bjursell and Reichard 1973). The mechanism appears to be allosteric (Bjursell and Reichard 1973). The mutagenicity of deoxythymidine and 5'-bromodeoxyuridine could result from an alteration of the intracellular nucleotide pools. Deoxythymidine and deoxycytidine are believed to act in this way (Bradley and Sharkey 1978 and Saffhill and Abbot 1979). Deoxythymidine and 5'-bromodeoxyuridine may act by starving the cell for deoxycytidine which results in the misincorporation of deoxythymidine (or 5'-bromodeoxyuridine)

for deoxycytidine in cellular DNA (Bradley and Sharkey 1978 and Kaufman Davidson 1978). However both deoxythymidine and 5'-bromodeoxyuridine cause mutagenesis in the presence of high concentrations of deoxycytidine, so their action cannot result from the starvation of the cell for deoxycytidine (Davidson and Kaufman 1978). The ability of deoxycytidine to reverse some of the effects of 5'-bromodeoxyuridine has recently been shown to be dependent on the conversion of deoxycytidine to deoxythymidine. However, the inhibition of the effect of 5'-bromodeoxyuridine is not a result of changes in the substitution of 5'-bromodeoxyuridine for thymidine in cellular DNA (Davidson and Kaufman 1977 and Kaufman and Davidson 1978). It seems more likely that mutagenesis is caused by alterations in the relative concentrations of the nucleotide triphosphates. It has been suggested that increases in the dTTP/dCTP ratio are mutagenetic, while changes in the dATP/dGTP ratio are merely toxic. The inhibitor for DNA synthesis, hydroxyurea, is known to change the latter ratio (Bradley and Sharkey 1978). There are fluctuations in the levels of all the deoxynucleotides during the cell cycle, but studies on synchronised cells have shown that the dCTP pool changes are the only ones that closely correlate to the rate of DNA synthesis (Bjursell and Reichard 1973 and Skoog, Nordenskjold and Bjursell 1973).

Another possible role for 5'-bromodeoxyuridine induced mutagenesis is by the induction of error-prone DNA repair, or by increasing the frequency of error by DNA polymerase itself (Kaufman and Davidson 1978).

Interferon yields have been reported to be increased after pretreatment of various cells with 5'-bromodeoxyuridine prior to induction (Tovey *et al* 1977). The following series of experiments seeks to provide information on the mechanism of action of 5'-bromodeoxyuridine in modulating the interferon system in Namalwa cells.

Results.

Firstly optimisation of the conditions for the induction of interferon using 5'-BrdUrd pretreatment was investigated. Initially 72 hours pretreatment was used prior to induction of Namalwa cells with Sendai virus in the absence of 5'-BrdUrd, as published (Tovey et al 1977). A table consisting of two experiments made to optimise all the variables (Table 6) shows several points. Firstly 5'-BrdUrd is toxic to the cells since their growth is inhibited to greater extents according to the dose of 5'-BrdUrd used (fig.9 and Table6A). An increase in the yield of interferon from treated compared to untreated cells is also seen. The effects of cell density and quantity of inducer are unaltered by 5'-BrdUrd (compare Table 6B & C to fig.2 & Table 1). Further experiments (Table 7) confirmed the optimal conditions to be 1.0×10^6 cells/ml induced with $100HA/10^6$ cells in 2% serum after pretreatment with 25ug/ml of 5'-BrdUrd. No interferon is detected in the absence of induction.

It was found that a shorter period of pretreatment resulted in higher titres of interferon (Table 8). 48 hours pretreatment was chosen as giving the most consistent yields since 24 hours pretreatment gave much lower titres in preliminary experiments (Table 8). This suggests that there is a lag period before 5'-BrdUrd can increase yields of interferon. Further experiments were done to investigate whether 5'-BrdUrd needs to be incorporated into cellular DNA before interferon yields are increased.

Other analogues of thymidine that differ from 5'-BrdUrd only in the substitution of another halogen atom for bromine at the 5' position of the molecule were tested (fig.10). Cells from the same culture were pretreated for 72 hours with each analogue. The analogues other than 5'-BrdUrd are more toxic than 5'-BrdUrd and FdUrd inhibits all cell division (Table 9). In the latter case most of the cells were still viable as judged by trypan blue exclusion. The interferon yields produced after pretreatment and induction under the optimal conditions judged for 5'-BrdUrd are shown in (fig.10). CldUrd is as efficacious as 5'-BrdUrd while IdUrd and FdUrd are less active.FdUrd indeed depresses interferon yields. It is known that IdUrd isincorporated less well into DNA as a thymidine analogue compared to 5'-BrdUrd and FdUrd is not incorporated at all. Although this data

Table

Pretr
(µg/ml 5
A)

1
2
5
10

B)

C)

D)

Table Legend.

A) Variable concentration of 5'-BrdUrd. Induction done with 100 HAU Sendai virus/10^6 cells, 10^6 cells/ml and 2% serum.

B) Variable cell density during pretreatment. Induction as A.

C) Variable Sendai virus concentration during induction. Induction with 10^6 cells/ml in 2% serum.

D) Variable serum throughout. Induction with 10^6 cells/ml and 100 HAU Sendai virus/10^6 cells.

Table 6. Optimisation of the conditions for pre-treatment and induction of Namalwa cells for interferon.

Pretreatment (μg/ml 5'-BrdUrd)		Cell density ($\times 10^6$ cells/ml)	Interferon yield ($\log_{10}$ U/ml/10^6 cells)
A)			
0		0.85	2.9
5		0.80	3.4
10		0.79	3.1
25		0.77	3.7
50		0.68	3.3
100		0.63	3.6
B)	cells/ml		
25	0.5×10^6	0.59	2.5
	1.0		2.5
	2.0		3.2
	5.0		3.2
	10.0		3.2
C)	Hz/ml		
25	1.3	0.64	1
	6.5		2.7
	13		2.6
	65		3.2
	130		2.8
	485		3.3
	1000		3.2
D)	% serum		
25	0	0.70	3.7
	1	0.70	4.1
	2	0.77	3.7
	5	0.88	2.9
	10	0.99	3.2

n done with
and 2% serum.

duction as A.

nduction.

^{6} cells/ml

Figure 9.

The cytotoxicity of 5'-bromodeoxyuridine.

Cell density of Namalwa cells (initially 5×10^5 cells/ml) after 72 hours in the presence of various concentrations of 5'-bromodeoxyuridine.

Averages of four determinations.

s/ml) after

Table 7. Yields of interferon from Namalwa cells. The effect of 5'-bromodeoxyuridine.

Pretreatment* (ug/ml 5'-BrdUrd)	Cell density ($x10^6$ cells/ml)	Serum %	Interferon yield log 10 U/ml/10^6 cells (1)**	(2)**
25	1.0	1	3.7	2.5
		2	2.5	2.7
25	2.0	1	2.3	2.8
		2	2.2	3.0
50	1.0	1	2.4	2.6
		2	2.8	2.0
50	2.0	1	2.4	2.9
		2	2.0	2.0

* Pretreated (see methods) with 5'-BrdUrd for 72 hours before induction.

** (1) Induction with 100 HAU/10^6 cells of Sendai virus.
(2) Induction with 500 HAU/10^6 cells of Sendai virus.

Table 8. Optimisation of the time required for pretreatment of Namalwa cells with 5'-bromodeoxyuridine.

A) Interferon Yields (log 10 U/ml/10^6 cells)

Pretreatment (ug/ml 5'-BrdUrd)	Pretreatment Time (hours)				
	24	24*	48	48**	72
0	2.2	3.8	4.2	3.8	3.7
5	2.0	4.6	4.4	3.7	4.3
10	2.8	4.7	4.7	3.8	4.4
25	2.2	4.6	4.7	3.7	4.4
50	2.7	5.0	4.3	3.0	4.8
100	2.8	4.5	4.1	4.1	4.6

B) Cell densities*** (x10^6 cells/ml)

Pretreatment (ug/ml 5'-BrdUrd)	Pretreatment Time (hours)				
	24	24*	48	48**	72
0	0.84	0.87	1.15	0.68	1.2
5	0.67	0.80	0.96	0.82	1.0
10	0.61	0.63	1.17	0.78	1.1
25	0.63	0.73	0.70	0.73	0.83
50	0.62	0.37	0.74	0.96	1.0
100	0.51	0.67	0.45	0.85	0.62

* Cells grown 24 hours, then pretreated with 5'-BrdUrd for 24 hours.

** Cells grown 48 hours, then pretreated with 5'-BrdUrd for 24 hours.

*** Prior to induction.

Means of duplicates.

Figure 10.

The effect of thymidine analogues on the interferon production from Namalwa cells.

Interferon yields from Namalwa cells after 48 hours pretreatment with varying concentrations of 5'-BrdUrd (● - ●), CldUrd (O-O), IdUrd (□ - □) or FdUrd (■ - ■).

Mean of two experiments (duplicate determinations).

Interferon yield ($\log_{10}$ U/ml)
5
4·5
4
3·5
3
5
10
25
50
75
100
Concentration of analogue (μg/ml)

(●–●),

Table 9. The effect of thymidine analogues on the growth of Namalwa cells.

Cell densities (x 10^6/ml)

Pretreatment (ug/ml)	FdUrd	CldUrd	5'-BrdUrd	IdUrd
0	0.82	0.82	1.2	ND
5	0.50	0.62	1.0	0.87
10	0.49	0.62	1.1	0.73
25	0.55	0.68	0.83	0.78
50	0.56	0.54	1.0	0.69
100	0.44	0.51	0.62	0.54

Mean of two experiments (duplicate determinations).

indicates that incorporation of 5'-BrdUrd into DNA may be a pre-requisite for increased interferon yields, it is still possible that other cellular sites with some structurally specific receptor(s) may be the target.

If the cells are pretreated with thymidine alone, (fig.11) there is no comparable increase in interferon yields to 5'-BrdUrd pretreatment. Thymidine can be used to compete out the effect of 5'-BrdUrd (fig.12). The higher the proportion of thymidine the greater the reduction of the effect of 5'-BrdUrd. It seems most probable that thymidine is competing for incorporation into DNA and thus depressing the quantity of 5'-BrdUrd in DNA.

Thymidine can also compete out acid precipitable incorporation of labelled 5'-BrdUrd (fig.13). The amount of exogenous thymidine incorporated varies little in the presence of variable amounts of 5'-BrdUrd. This may reflect changes in the cellular pool size of available thymidine molecules in response to uptake of exogenous thymidine. This may also result from the differential rate of incorporation of 5'-BrdUrd to thymidine (fig.14). This may be the result or cause of the different pool sizes of the nucleotides, qualitatively indicated by the determination of the acid soluble radioactivity. The technique of caesium chloride density gradient centrifugation was used to indicate whether cellular DNA was the site of action of 5'-BrdUrd. It was shown that about 50% of the thymidine in cellular DNA was replaced by 5'-BrdUrd when cells were cultured in the presence of 25ug/ml 5'-BrdUrd (fig.15).

When 5'-BrdUrd is added to saturation density cells, which grow only slowly, there is no increase in interferon yields comparable to that seen with the effect of 5'-BrdUrd on growing cells (Table 10). Addition of deoxycytidine to the medium, should present the cytotoxic effect by correcting the deficienoy in deoxycytidine resulting from 5'-BrdUrd treatment (see introduction). A reversal of this cytotoxic effect of 5'-BrdUrd does occur with deoxycytidine up to 48 hours (Table11) without any change in the enhanced interferon yields following 5'-BrdUrd pretreatment (Table 10). Deoxycytidine had no effect on the yields of interferon produced in 5'-BrdUrd treated saturation density cells (Table 12).

Figure 11.

The effect of thymidine on Namalwa cells.

The effect of thymidine on
a) interferon production in Namalwa cells.
(Å-Δ) pretreatment with 5'-BrdUrd.
(O-O) pretreatment with thymidine.

b) growth of Namalwa cells.
(•-•) pretreatment with 5'-BrdUrd.
(o-o) pretreatment with thymidine.

The mean of two determinations.

Fig 11a.

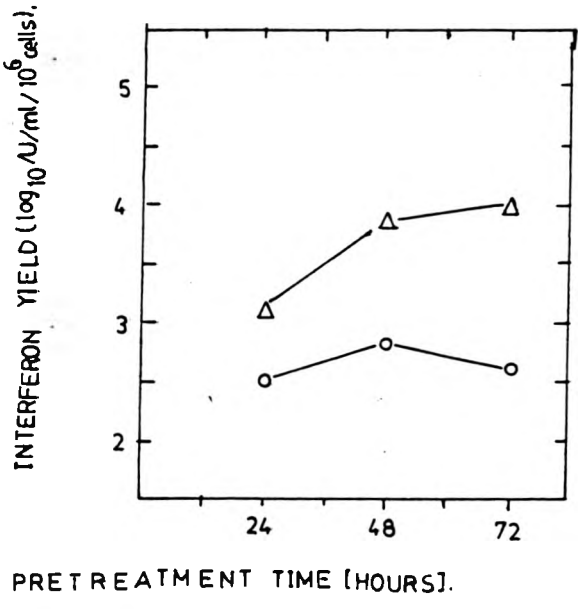

INTERFERON YIELD (log_{10} U/ml/10^6 cells).
5
4
3
2
24
48
72
PRETREATMENT TIME (HOURS).

Fig 11b.

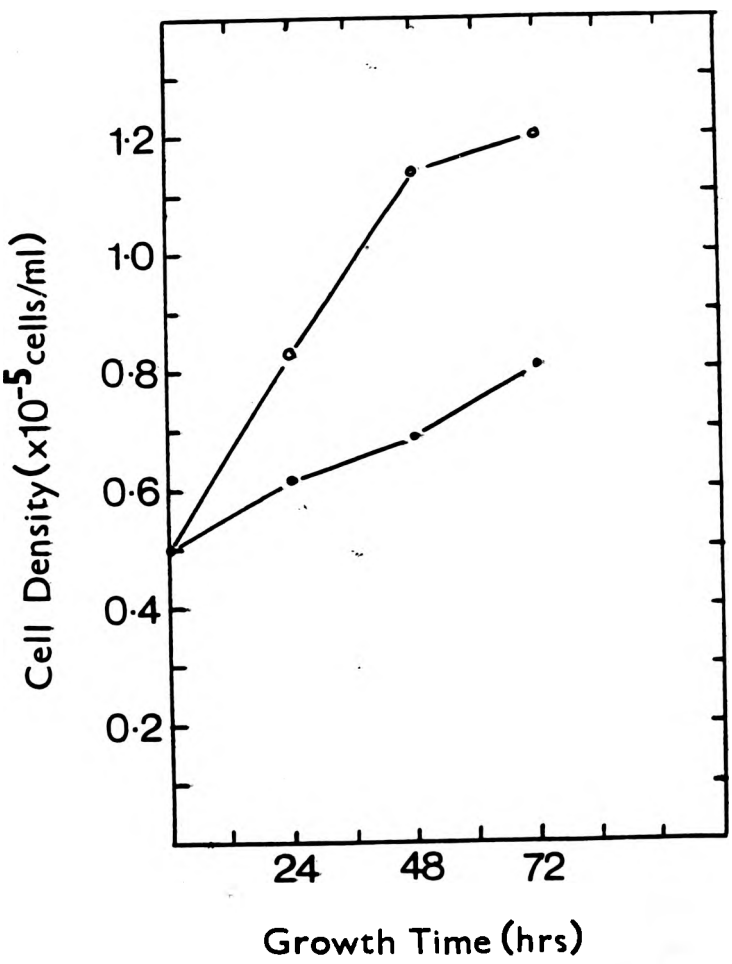

1·2
1·0
0·8
0·6
0·4
0·2
Cell Density (x10⁻⁵cells/ml)
24
48
72
Growth Time (hrs)

Figure 12.

The effect of thymidine competition on the stimulation of interferon in Namalwa cells by 5'-BrdUrd.

5'-BrdUrd as a percentage of the total concentration of additives (81.4mM).

Means of two determinations.

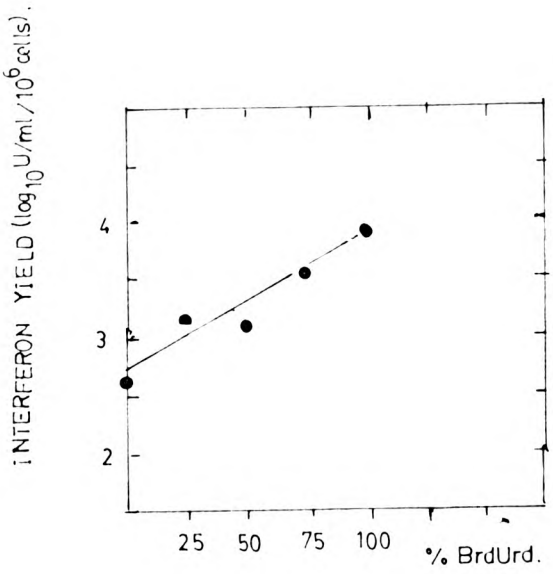

INTERFERON YIELD (log_{10}U/ml./10^6 cells).
4
3
2
25
50
75
100
% BrdUrd.

Figure 13.

The effect of thymidine competition on the uptake of 5'-bromodeoxyuridine by Namalwa cells.

Namalwa cells labelled with either 2.5μCi/ml ^{3}H-BrdUrd or ^{3}H-thymidine.

a) The incorporation of ^{3}H-BrdUrd with increasing thymidine concentration (●–●).

b) The incorporation of ^{3}H-thymidine with increasing 5'-BrdUrd concentration (O–O).

c) Comparison of a) and b) with corrections for the specific activity of the radiolabels

^{3}H-BrdUrd (●–●).

^{3}H-thymidine (O–O).

Means of 3 experiments with duplicate determinations.

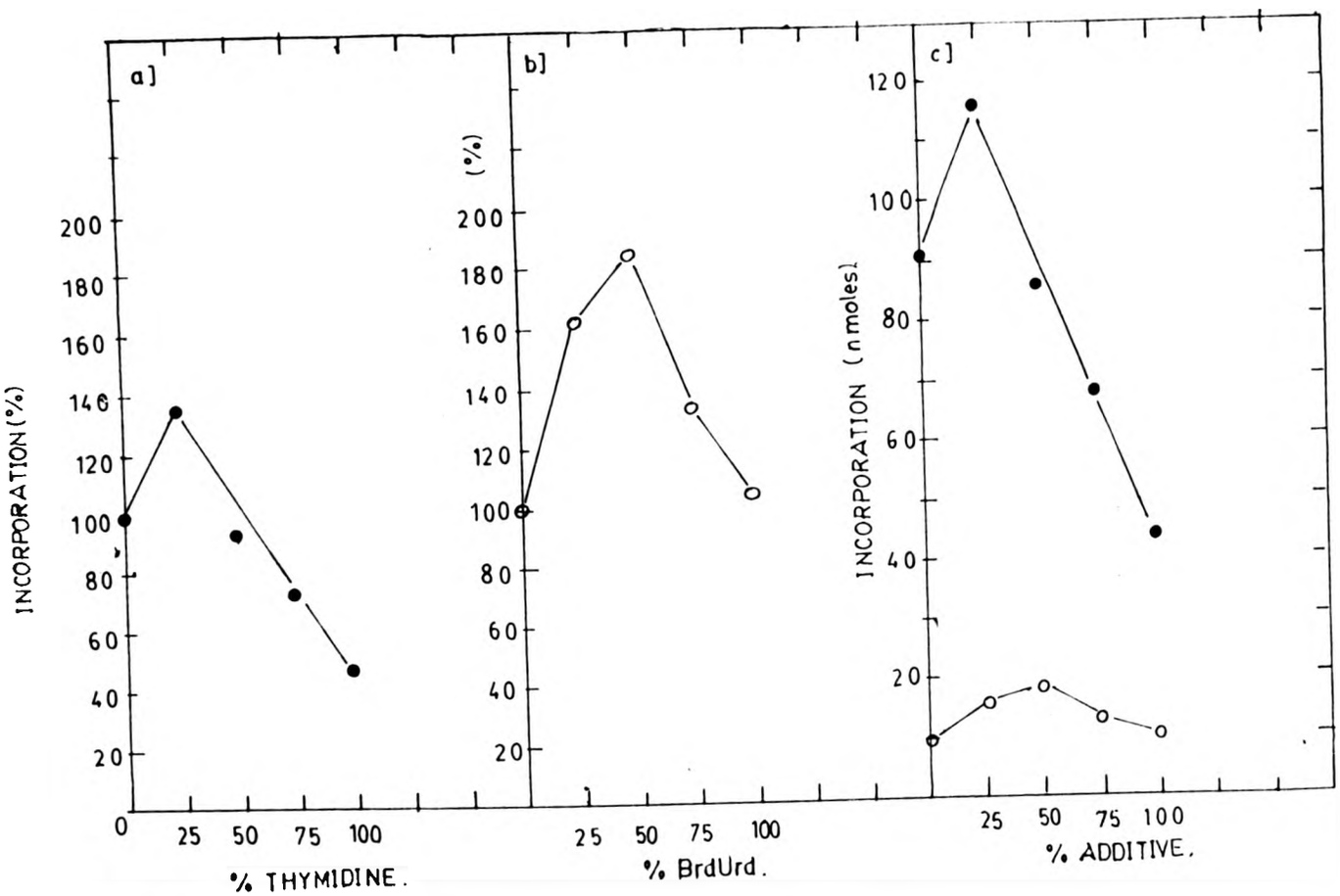

a]
b]
c]
INCORPORATION (%)
(%)
INCORPORATION (nmoles)
200
180
160
140
120
100
80
60
40
20
0
25
50
75
100
% THYMIDINE.
% BrdUrd.
% ADDITIVE.

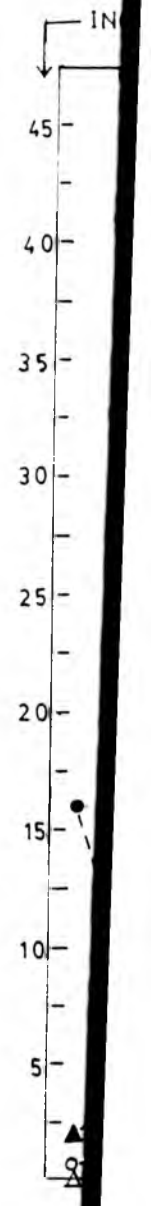
Figure 14.

The rate of incorporation of thymidine and 5'-bromodeoxyuridine into Namalwa cells.

Namalwa cells labelled with either 5μCi/ml ^{3}H-BrdUrd or ^{3}H-thymidine.

The kinetics of incorporation of ^{3}H-BrdUrd (▵ - ▵) and ^{3}H-thymidine (▲ - ▲) into acid precipitable material and determinations of the concommitant acid soluble ^{3}H-BrdUrd (•-•) and ^{3}H-thymidine (o-o) from Namalwa cells.

Means of three experiments with duplicate determinations.



or

ad
and

lwa cells.

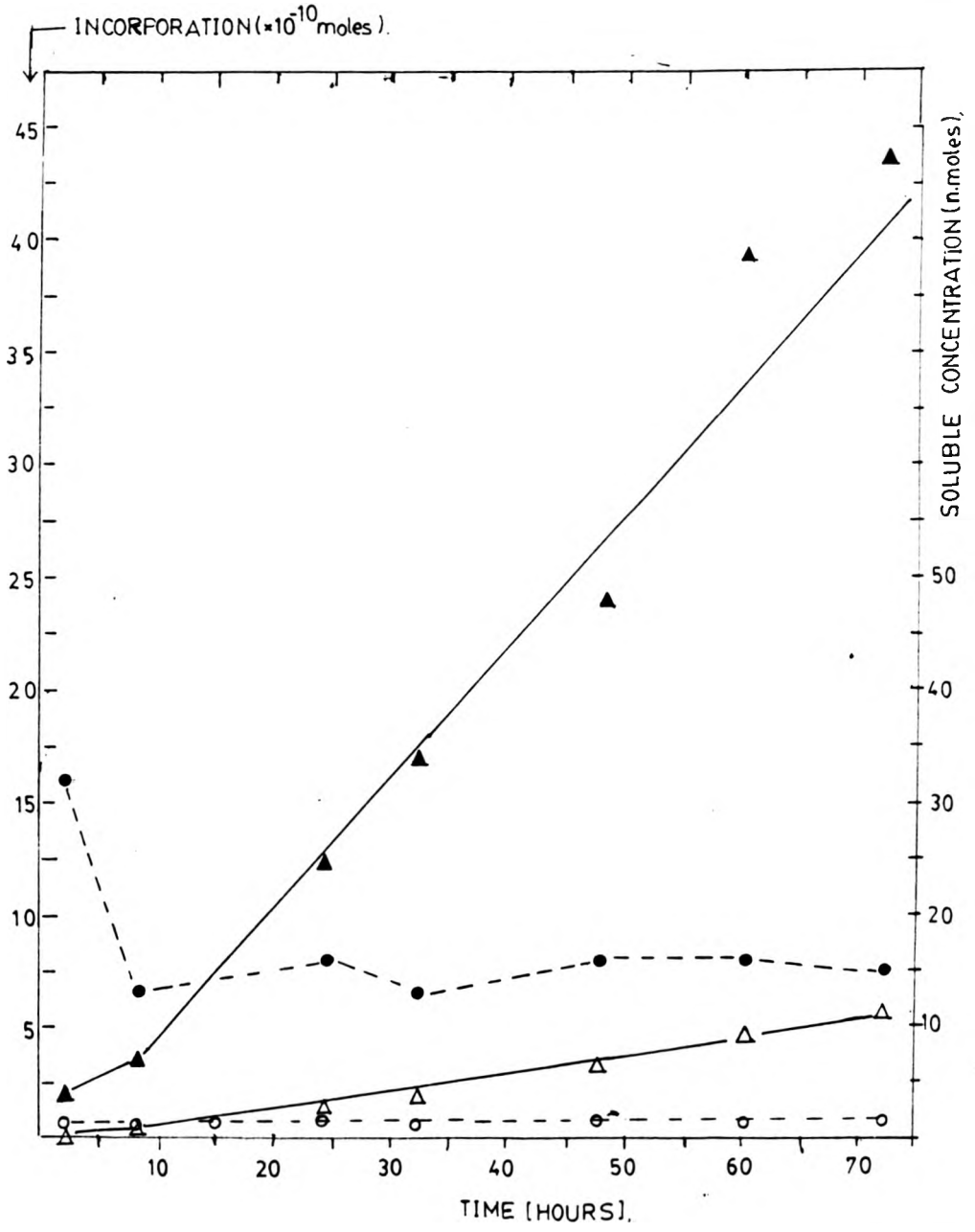
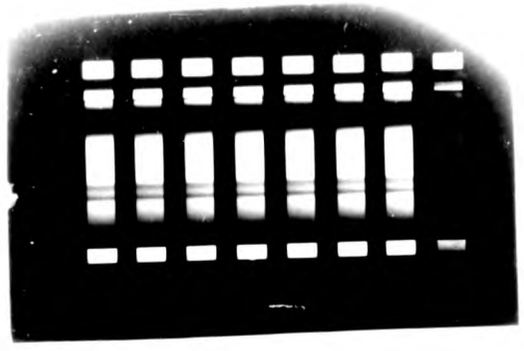
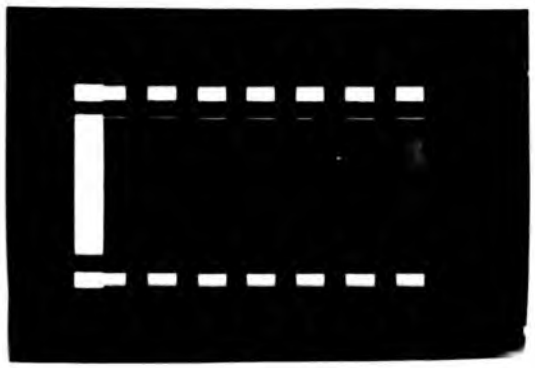


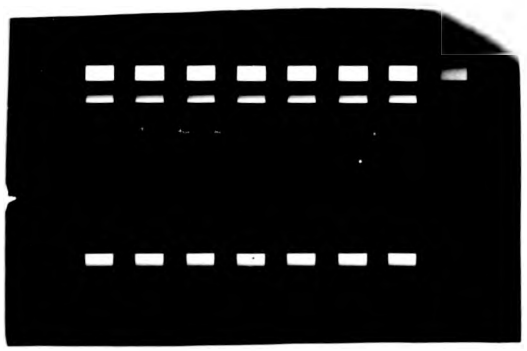
Figure 15.

The incorporation of 5'-bromodeoxyuridine into cellular DNA.

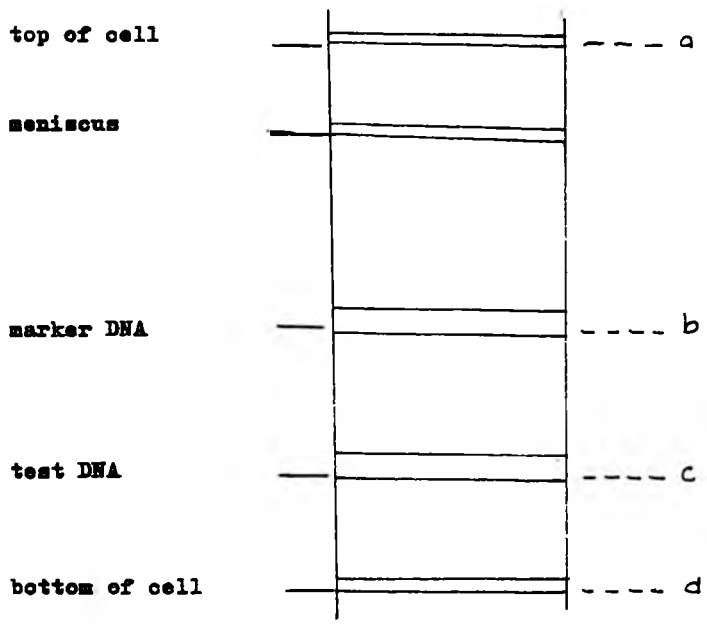




a) Unsubstituted DNA and marker



b) Substituted DNA and marker.



density of CsCl = 1.4010, marker DNA = 1.7100.

length of cell = 1.4cm.

Magnification of photograph = $\frac{a-d}{1.4}$ = 7.6/1.4 = 5.43.

Distance of sample band from centre of rotation (r_s) = dist. of rotor centre to a + $\frac{a-c}{\text{magnification factor.}}$

$$r_s = 5.71 + \frac{5.65}{5.43}.$$

$$r_s^2 = 45.57.$$

Similarly for marker $r_m = 5.71 + \frac{a-b}{5.43}$.

$$r_m^2 = 41.09.$$

$\rho_s = \rho_m + 4.2w^2 (r_s^2 - r_m^2) \times 10^{-10}$ g/cm^3 at 44,000 rpm.

$w_2 = 2.123 \times 10^7$, $4.2w^2 \times 10^{-10} = 8.9166 \times 10^{-3}$.

$\rho_s = \rho_m + 8.9166 \times 10^{-3} (r_s^2 - r_m^2)$

$\rho_s = 1.71 + 8.9166 \times 10^{-3} (45.57 - 41.09)$

$= 1.75$g/cm^3.

Similarly for Namalwa DNA unsubstituted with 5'-BrdUrd.

a - b = 5.65cm.
a - c = 5.15cm.

magnification factor = $7.8/_{1.4}$ = 5.57.

r_s^2 = 44.02.

r_m^2 = 45.22.

ρ_s = 1.6993.

ρ 5'-BrdUrd = 1.75.
ρ unsubst. = 1.699.

using GC = $\frac{\rho - 1.660}{0.098}$ then the mole fraction of 5'-BrdUrd present in cellular DNA = 0.15.

Since thymidine has a mole fraction of about 0.3 in unsubstituted DNA this suggests about 50% of thymidine residues are replaced with 5'-BrdUrd.

Macromolecular synthesis in treated cells compared to untreated cells was studied by following the incorporation of labelled precursors, (uridine, methionine and thymidine), into acid precipitable material, in the presence and absence of 5'-BrdUrd. Fig. 16a and b shows that uridine and methionine incorporation in Namalwa cells, whether infected or uninfected, is only slightly depressed in the presence of 5'-BrdUrd. However thymidine incorporation is significantly inhibited (fig.16c).

The kinetics of interferon production with 5'-BrdUrd show that there is merely more interferon produced at any given time compared with untreated cells (fig.18). This contrasts with the changes in the kinetics of production in response to antimetabolites, referred to as superinduction. Also no Sendai virus appears to be made in treated cells, thus Sendai virus replication is not enhanced in the manner in which latent virus production is induced by 5'-BrdUrd.

Discussion.

The results show that pretreatment of Namalwa cells with the thymidine analogue 5'-BrdUrd results in higher titres of interferon if the cells are induced with Sendai virus. This phenomena is not akin to 'superinduction' as described for interferon in fibroblast cells as judged by the kinetics. Thymidine alone does not result in higher titres of interferon and it competes out the effect of bromodeoxyuridine. 5'-BrdUrd gives rise to toxicity in the cells. This presumably occurs by alterations in the deoxynucleotide pools, particularly that of deoxycytidine, in the manner described for other systems (see introduction). DNA synthesis is inhibited by 5'-BrdUrd and this may result from the pool changes. Deoxycytidine reverses the toxic effect to some extent without reversing the effect of 5'-BrdUrd on interferon titres. Thus it seems likely that 5'-BrdUrd is incorporated into DNA as a prerequisite for its effect on interferon production. It is incorporated in 50% of the DNA in place of thymidine by 48 hours. Thus if a single cell division had occurred (the doubling time being 30 hours) all the substitution would be in a single strand of DNA and thus represents total substitution. This may be why 48 hours is the optimum time for pretreatment.

Table 10. The effect of deoxycytidine on interferon yields in Namalwa cells.

Pretreatment* (μg/ml 5'-BrdUrd)	dc**(μM)	Interferon Yield (log_{10}U/ml/10^6 cells) Growing cells		Saturation density cells	
0		(0.92)***	2.8	(1.7)	3.25
25		(0.75)	3.6	(1.7)	3.55
50		ND		(1.5)	3.3
100		ND		(1.8)	3.5
0	100	(0.85)	3.05	ND	
25	10	(0.98)	3.6	ND	
25	100	(0.80)	3.65	ND	
25	500	(1.00)	3.35	ND	
50	100	(0.93)	3.35	(1.5)	3.35

* 48 hours with one or both 5'-bromodeoxyuridine and deoxycytidine.

** Deoxycytidine.

*** Cell density prior to induction.

ND Not done.

Means of three experiments.

Table 11. The effect of deoxycytidine on the toxcity of 5'-bromodeoxyuridine.

Cell density (x 10^6/ml)

Pretreatment		Pretreatment time (hours)		
5'-BrdUrd(μg/ml)	dC*(μM)	24	48	72
0	0	0.44	0.73	0.96
0	0	0.56	0.65	0.87
25	0	0.31	0.54	0.64
25	0	0.31	0.49	0.66
25	10	0.40	0.72	0.80
25	100	0.43	0.65	0.73
25	100	0.42	0.60	0.74
25	500	0.45	0.56	0.64
0	100	0.44	0.69	0.96
0	500	0.44	0.77	0.74

* Deoxycytidine.

Means of three experiments.

Table 12. The effect of deoxycytidine on interferon production in stationary Namalwa cells.

Pretreatment*		Interferon Yield
5'- BrdUrd (μg/ml)	dC** (μM)	($\log_{10}$U/ml/10^6 cells)
0	0	3.5
0	100	2.9
0	100	2.8
25	0	3.1
25	0	3.2
50	0	3.0
100	0	3.3
25	10	2.75
25	100	2.5
25	500	2.6
50	100	3.05

* 48 hour.

** Deoxycytidine.

Means of three experiments.

Figure 16.

The effect of 5'-bromodeoxyuridine on cellular macromolecular synthesis.

The incorporation of ^{35}S-methionine a) ^{3}H-uridine b) and ^{3}H-thymidine c).

O–O Untreated TCA precipitate.

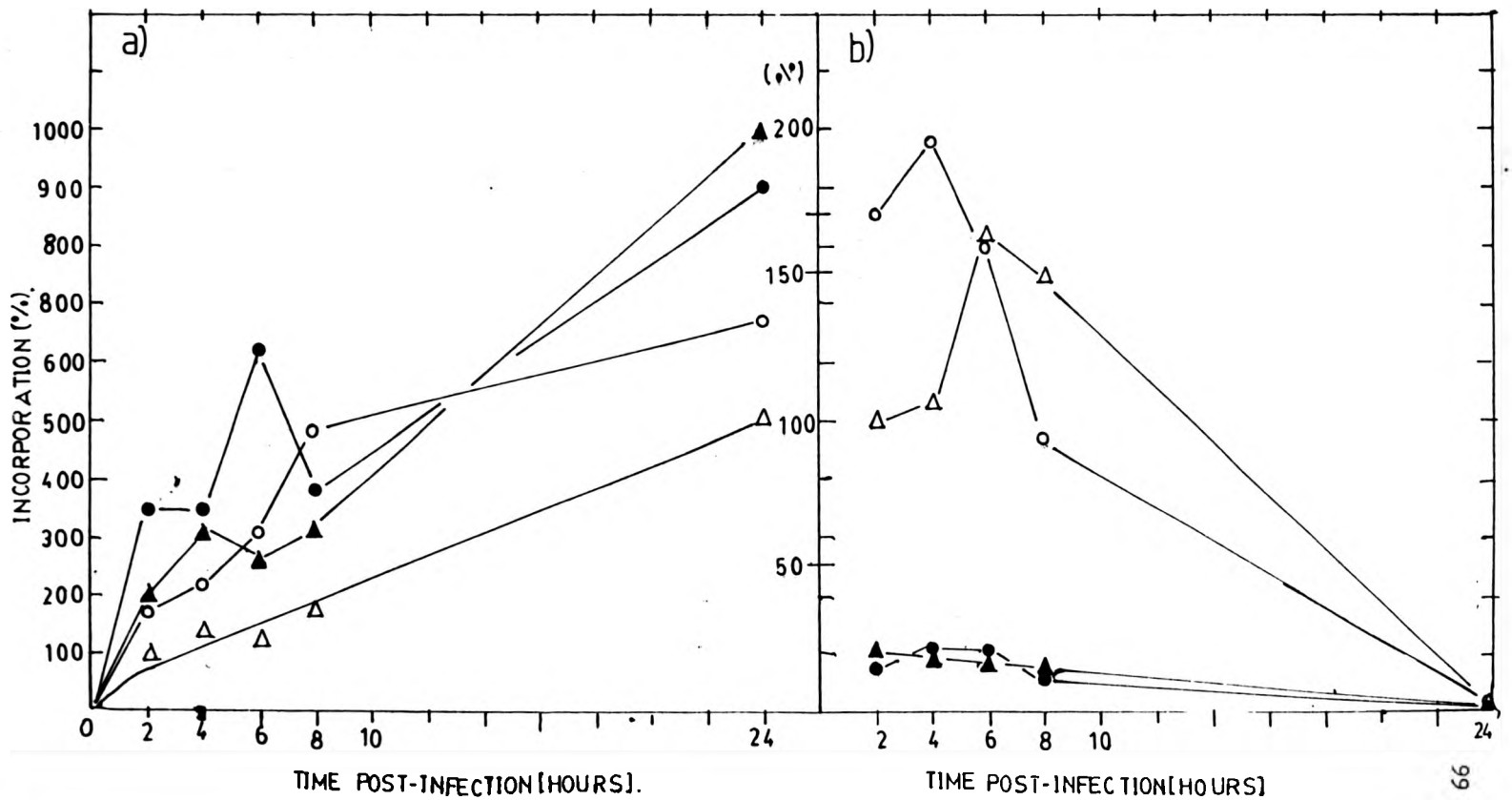
Δ-Δ Treated TCA precipitate.

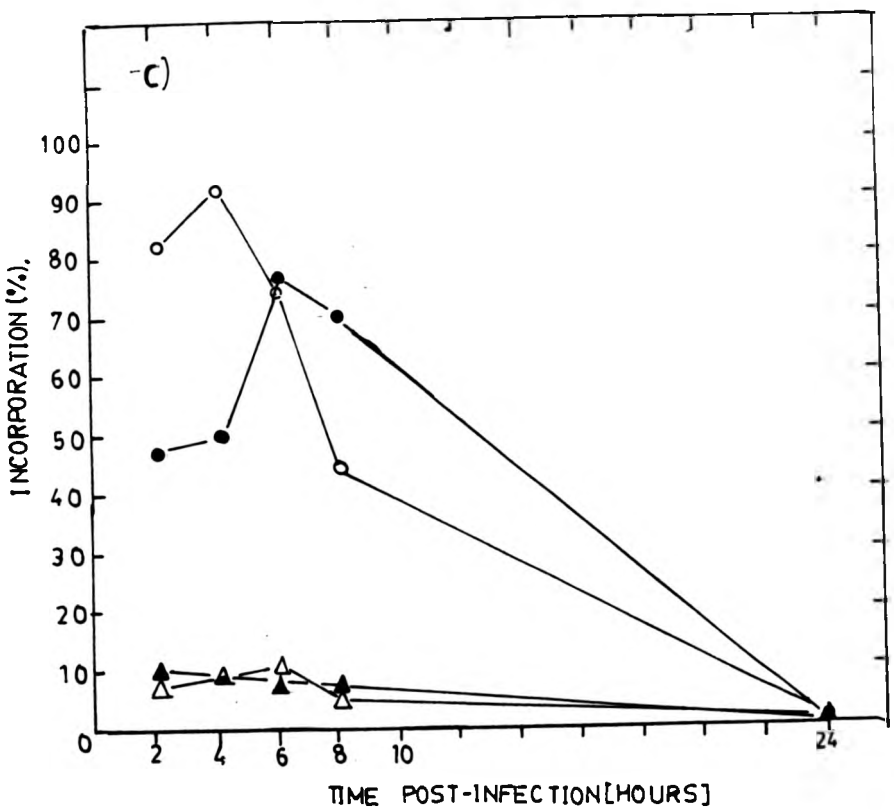
●–● Untreated TCA soluble.

▲-▲ Treated TCA soluble.

Incorporation determined relative to the initial determination (=100%).

Mean of three determinations.


a)
b)
INCORPORATION (%).
1000
900
800
800
600
500
400
300
200
100
0
2
4
6
8
10
24
TIME POST-INFECTION [HOURS].
200
150
100
50
TIME POST-INFECTION [HOURS]


-C)
100
90
80
70
60
50
40
30
20
10
0
INCORPORATION (%).
2
4
6
8
10
24
TIME POST-INFECTION[HOURS]

Evidence to support DNA as the site of action are the following: FdUrd behaves in a manner opposite to that of 5'-BrdUrd and is not incorporated into DNA. 5'-BrdUrd is not effective in non-growing cells. Labelled 5'-BrdUrd is also competed out of acid precipitable material (DNA) by thymidine as is the effect of 5'-BrdUrd.

The work presented in this section thus far represents my work with 5'-BrdUrd on Namalwa cells. These results are incorporated in a paper (see Appendix 1) where they are paralleled by a study of 5'-BrdUrd on fibroblast cells undertaken by Dr. T. K. Bradshaw. It seems pertinent to briefly discuss these results since they in part support and in part modify the conclusions presented above.

The cells used for this work were a normal fibroblast line HFF and an SV40 transformed line of fibroblasts WI-26. 5'-BrdUrd had a similar, though smaller, effect on interferon production after induction of HFF cells but had no effect on the WI-26 line. 5'-BrdUrd was cytotoxic to both these lines but even when the toxicity was reversed using deoxycytidine, interferon titres were not increased in the WI-26 cells. 5'-BrdUrd, however, was found to be incorporated into both these cell types to a similar extent compared to Namalwa cells during the period required to attain confluency and maximal interferon titres. Thus it is suggested that more than just the incorporation of 5'-BrdUrd into cellular DNA is required before there is a stimulation of interferon production. It should be noted, however, that the SV40 transformed WI-26 line can only be induced with poly(rI).(rC) and not with virus, whereas this situation is reversed for Namalwa cells. The effect of 5'-BrdUrd on interferon production in HFF cells can be demonstrated regardless of the manner of induction (poly(rI).(rC), NDV or superinduction). This suggests the action of 5'-BrdUrd is independant of the manner of induction. Thus the block preventing the SV40-WI-26 line cannot be ascribed to differences in induction. It is not known if some other action of 5'-BrdUrd is required to stimulate interferon production or whether its activity is determined by the host cell.

As far as the DNA linked part of the mechanism is determined for Namalwa cells, similar results are obtained for HFF cells.

Thymidine itself is not stimulatory and competes out the effect of 5'-BrdUrd. In confluent HFF cells where DNA synthesis is depressed to low levels, 5'-BrdUrd has no effect on interferon production. This may reflect a low incorporation of 5'-BrdUrd into cellular DNA. Deoxycytidine reversed the growth inhibition due to 5'-BrdUrd without affecting the stimulation of interferon production. Thus similar conclusions to that present for Namalwa cells, can be drawn from this cell line. In addition it can be pointed out that a stimulation of interferon production after 5'-BrdUrd treatment occurred when HFF cells were superinduced. Thus it seems the mechanism of action of 5'-BrdUrd is distinct from the actions of the antimetabolites used in superinduction.

Possibly the mode of action of 5'-BrdUrd reflects the hypothesis put forward in the introduction whereby an alteration in the binding of chromosomal proteins occurs resulting in changes in cellular gene regulation. One would predict from this model that increased transcription of the interferon gene results from 5'-BrdUrd treatment. Clearly the short time required for the effect precludes any mechanism based on the direct mutangenic effect of 5'-BrdUrd, though changes in the deoxynucleotide pool sizes may occur and could be responsible for the inhibition of DNA synthesis and growth.

IV. Butyric acid - a modulator of gene expression.

The use of sodium butyrate as a control for the effects of dibutyryl-cyclic adenosine monophosphate (dcAMP) lead to the discovery of morphological and biochemical changes in mammalian cells by this agent alone. Some of these effects are similar to those caused by dcAMP and are attributed to cAMP as a general cell effector. Other effects are unique to sodium butyrate (Prasad and Sinha 1976). Only butyric acid and a few closely related short chain fatty acids are active.

HeLa cells treated with sodium butyrate show hugh morphological changes, including the formation of very long processes. Electron microscopy shows that the cell membranes remain intact and there is no change in the number of several cell organelles (Deutsch et al 1976). Actinomycin D and Cycloheximide inhibit the morphological changes including the formation of the processes, so protein synthesis seems to be required for their formation, but once formed very little biosynthetic activity is required to maintain them (Ginsberg et al 1973 and Henneberry and Fishman 1976). There are large increases in micro-filament bundles and cytochalasin B and colchicine - inhibitors of microfilament and microtubule formation respectively - inhibit process formation. Thus microfilament and microtubule arrangement may be required for process formation (Ghosh et al 1976 and Altenburg, Via and Steiner 1976). Cytochalasin B has been observed to inhibit protein synthesis and break down polyribosomes (Koch and Oppermann 1975).

Thus, like many inhibitors, cytochalasin B and colchicine are not specific enough to draw conclusive results from their use. This has not stopped speculation that sodium butyrate might specifically induce or alter the synthesis or activity of a microtubule assembly protein analogous to the tau protein isolated from porcine brain and identified in many cell cultures (Henneberry and Fishman 1976 and Lockwood 1978). Mono dibutyryl cAMP also causes this type of morphological change in chinese hamster ovary cells and microtubules are again implicated (Hsie and Rick 1971, Henneberry, Fishman and Freese 1975). Regulation of the intracellular calcium ion concentration by the use of a specific calcium ionophore, also suggests a role for calcium in these morpho-

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logical changes (Henneberry, Fishman and Freese 1975), especially since calcium has an apparent regulatory role in microtubule formation (Borisy et al 1974 and Gallin and Rosenthal 1974). Treatment of certain cells with sodium butyrate can enhance or depress the activity of several enzymes. Examples of enhanced activity are: tyrosine hydroxylase, choline acetyltransferase, acetylcholine-esterase, catechol-methyl transferase, adenylate cyclase, alkaline phosphatase, a 5'-nucleotidase and a specific siayl transferase. An inhibition of activity is seen for the glucose phosphorylating enzymes - glucokinase and hexokinase - tyrosine aminotransferase and melanin formation (see Prasad and Sinha 1976 for review). It has been concluded that only membrane bound enzymes are altered in activity, while those enzymes from other putative subcellular locations remain unaltered (Deutsch et al 1976).

It seems that the increase in siayl transferase activity in HeLa cells is important in the morphological change seen (Simmons et al 1975 and Henneberry and Fishman 1976). Butyrate also causes changes in the glycolipids of the cell membrane (Simmons et al 1975) and an incorporation of sodium butyrate itself into the cell membrane causes a change in membrane configuration (Deutsch et al 1975). Thus it seems that the membrane may be an important site for the action of sodium butyrate.

The induction of two enzymes - alkaline phosphatase and siayl transferase - in HeLa cells shows different kinetics. Colcemid treatment of HeLa cells in the presence of sodium butyrate can also decrease the activity of alkaline phosphatase but not of siayl transferase. Thus it has been suggested that the two activities are altered by different mechanisms. The activity of siayl transferase may be governed at the level of transcription while the activity of alkaline phosphatase may be governed by its degree of phosphorylation (Deutsch et al 1976). The induction of siayl transferase is blocked by actinomycin D and cycloheximide, so new mRNA and protein synthesis are required supporting the theory that gene transcription may be altered in this case.

In HeLa cells sodium butyrate enhances the production of two hormones. The α-subunit of human choronic gonadotrophin (HCG) but

not the β-subunit, which confers biological activity - is stimulated (Ghosh 1976, 1977, Ghosh et al 1977 and Tralka et al 1979). Similarly human follicle stimulating hormone (FSH) production is increased (Ghosh 1977). Estradiol secretion is inhibited by contrast and progesterone and placental lactogen also inhibits DNA synthesis in HeLa cells. Growth inhibition has been reported in several cell lines and an inhibition of DNA synthesis and a cessation of cell division occurs in some of these (Bombik and Burger 1973, Schneider 1976, Hagopian et al 1977 and Marks and Rifkind 1978). The stimulation of HCG α-subunit and FSH in HeLa cells can occur when inhibitors of DNA synthesis alone are used e.g. hydroxyurea (Ghosh 1977 and Ghosh et al 1977). FSH production was synergistically affected in the presence of both hydroxyurea and sodium butyrate. Thus an inhibition of DNA synthesis of cell multiplication may be required for changes in gene expression. It is interesting to note in passing that the HeLa cell genome is depressed for both differentiated and oncofoetal protein expression by these agents.

Sodium butyrate, dimethylsulphoxide and other planar-polar molecules as inducers of differentiation.

The Friend erythroleukaemia cell has been used as a model system for terminal differentiation. In culture, murine erythroleukaemia cell (MELC) lines - derived from mouse spleen cells infected with the Friend virus complex, grow with a low rate of spontaneous differentiation, about 0.5%. In the presence of certain inducers a much greater proportion of the cells differentiate (Hawkins and Krantz 1975). This induced differentiation is very similar to the normal differentiation, but occurs in the absence of the normal regulatory hormone - erythropoeitin. The cells rarely complete differentiation, which is marked by the extrusion of the cell nucleus to give non-nucleated cells. Different patterns of gene expression from normal erythropoeisis are also seen (Kabat et al 1975). Markers for differentiation include: morphogenesis

(Friend et al 1971), accumulation of globin α and β mRNA, α and β -globin synthesis, increases in heme biosynthetic enzymes, changes in purine metabolism (Reem and Friend 1975), production of red cell membrane proteins and a limited cell division capability (Kabat et al 1975).

Inducers of this differentiation include planar-polar compounds, like hexamethylene bisacetamide, purines or their derivatives, hemin, short chain fatty acids such as sodium butyrate, inhibitors of DNA or RNA synthesis and X- or UV-irradiation. Not all these compounds act in the same manner (Gusella and Houseman 1976, Ross and Lautner 1976 and Nudel et al 1977a, 1977b). Variants can be isolated that are resistant to one or all the inducers while others can respond to only a few but not all inducers (Harrison et al 1978 and Ohta 1976). The selection of dimethyl sulphoxide (DMSO) resistant clones is enhanced by mutagenesis (i.e. using alkylating agents). Some variants remain stable while others revert to the normal phenotype. It is argued that these variants arise by mutation, in spite of the fact that one alkylating agent, ethyl-methyl-sulphonate , does not give rise to variants and the reversion rate is very high. Most DMSO resistant variants are non-inducible by DMSO for all differentiation markers, but two variants are inducible for the erythrocyte specific membrane protein, spectrin (Harrison et al 1978). This is an early marker for differentiation and shows that spectrin induction can be uncoupled from other aspects of differentiation, such as globin synthesis. In no DMSO resistant variant has globin synthesis been decoupled from hemin synthesis, so there is no evidence for regulation at the level of translation. An inhibitor of translation can, however, be found in MELC lysates. This inhibitor has been identified as a protein kinase that specifically phosphorylates the initiation factor eIF-2 and prevents the formation of the initiation complex required for translation. This is similar to the situation found in cell lysates made from interferon treated cells, (see page 5). What function, if any, this inhibitor has in the intact cell is not known (see Marks and Rifkind 1976 p.436).

One variant, which is resistant to induction, can be treated with the inducer plus hemin and terminal differentiation will then

proceed. This suggests that the variant is deficient in hemin synthesis (Harrison et al 1978).

Regulation at the level of transcription does occur. RNA synthesis during differentiation declines overall as early as 2 hours after induction but there is an increase in globin mRNA synthesis within 6-24 hours, depending on the inducer and culture conditions. Globin mRNA synthesis is detectable in isolated nuclei, taken from DMSO induced MELC cells, after only 5 minutes. Transcriptional activation is the most likely explanation, although post-transcriptional stability of the mRNA may be increased (Orkin and Swerdlow 1977). The pattern of α and β-globin mRNA accumulation, however, differs depending on the inducer. β-globin sequences in mRNA are detectable later than α-globin sequences and occur at a lower frequency in butyric acid and hemin induced cells and possibly in DMSO induced cells. Different inducers give rise to different accumulation of the β minor globin mRNA. The β major and β minor globin genes are closely linked and the protein sequence differs by only 6-10 amino acids, yet hexamethylene bisacetamide and DMSO induced cells accumulate different amounts of the two mRNA's and correspondingly the proteins. There is no evidence for any translational or post-translational regulation. In contrast butyric and proprionic acid induction gives rise to equal amounts of the β major and β minor globins. (Nudel et al 1977a and b).

Following induction, haemoglobin synthesis reaches very high levels. About 0.5-1% of the poly-A containing RNA(mRNA) on polyribosomes is globin mRNA and up to 10% of the soluble protein in the cell is haemoglobin representing about 25% of the total cellular protein being synthesized (Kabat et al 1975).

The inducers have demonstrable effects on other cell types and cause a difference in oncogenicity (Marks and Rifkind 1978). The inducers have been shown to penetrate the cell membrane and when prevented from being taken up (e.g. by binding the inducer covalently to glutathione), then differentiation is not induced (Levy et al 1975, Marks and Rifkind 1978 p.429). Induction by DMSO is also cell cycle specific, the cells requiring a period of pre-treatment in S phase (Levy et al 1975). The evidence suggests

that the planar-polar compounds that induce are flexible and have an optimal separation of 5-8 methylene groups between their polar groups. These inducers are also toxic to the cells but it is not known if this is related to their mode of action or an independent effect (Marks and Rifkind 1978).

Effects on the structure of DNA.

Changes in both the structure of DNA and in the chromosomal proteins are observed *in vitro* and *in vivo* with the inducers DMSO, hexamethylene bisacetamide and butyric acid. The sedimentation coeffecient of the cellular DNA changes after induction, suggesting that some change in structure has taken place. Differences greater than 50% in the quantity of 6 non-histone chromosomal proteins, 2 nucleoproteins and 3 cytoplasmic proteins are seen after DMSO treatment of MELC cells (Peterson and McConkey 1977). A new protein of 25,000 molecular weight has also been observed in MELC cells after treatment with DMSO or hexamethylene bisacetamide. This protein is not found in resistant variants or uninduced cells. The protein has similar properties to histone H1, but is not structurally related (Keppel, Allet and Eisen 1977, 1979). Changes are seen in the histones isolated from butyrate treated cells. The most obvious changes are seen for histones H3 and H4. It has been suggested that these changes result from an increase in the acetylation of the histones.

A large proportion of the histones appears to be totally acetylated and pulse label experiments with ^{14}C acetate support the idea that acetylation is increased (Hagopian *et al* 1977). The mechanism of this increased acetylation appears to be an inhibition of the histone deacetylase enzymes. There are many reviews on histone modifications, but the function of this post-translational modification is still not clear (Georgiev 1969, Stellwagen 1969, Hearst 1970, Johnston and Allfrey 1978). It does appear that acetylated histones are preferentially associated with actively transcribing chromatin (Berlowitz and Pallota 1972, Gorosky *et al* 1973, Levy-Wilson, Gsereset and McCarthy 1977, Johnston and Allfrey 1978 and Oberhauser *et al* 1978). There is a correlation between

histone acetylation and DNA-dependent RNA synthesis and acetylation preceeds or accompanies increases in RNA polymerase activity and RNA synthesis (Johnston and Allfrey 1978). Thus histone acetylation is considered an early event in derepression of genes and important for the control of transcription (Ruiz-Carillo, Wangh and Allfrey 1973) or chromatin assembly (Louie, Candido and Dixon 1973), though it may not be the only mechanism for the stimulation of RNA synthesis. There are changes in the amounts and patterns of histone acetylation associated with gene activation in lymphocytes, gene suppression in granulocytes, viral transformation, during differentiation and development, and after treatment of certain cells with steroid or peptide hormones or drugs (Johnston and Allfrey 1978). Numerous studies show that histone acetylation alters DNA conformation. Studies with reconstituted chromatin *in vitro* support this and show changes in template activity (Marushige 1976 and Wallace *et al* 1977).

Non-histone proteins are also involved in chromatin structure (Adolph *et al* 1977) and in gene regulation (Chiu and Hnilica 1976). Phosphorylated non-histones are particularly implicated. Phosphorylation of histones also occurs and is involved in alterations of chromatin structure and function (Johnston and Allfrey 1978). In at least one case a cAMP dependant protein kinase catalyses the *in vitro* phosphorylation of histone 2A at a specific serine (Shyaprukov *et al* 1975). Since some of the butyrate induced effects are mediated by cAMP, phosphorylation of chromosomal proteins is a possible mode of action, although there is no evidence to support this idea.

Work with non-erythroid systems show that DMSO can destabilise the thermostability of base-pairs and protein-DNA interactions *in vitro* (Lapeyre and Bekhor 1974). Specific changes in the gene expression of *E. coli* can be seen after treatment with DMSO, both *in vivo* and *in vitro* (Nakanishi *et al* 1974). It seems likely that the patterns of gene expression seen are a result of the specificity of the chromatin structure already present in the cell. One may consider the precursor erythroid cell as 'pre-programmed' and normally required only a single signal to initiate differentiation.

Since inducers of differentiation other than the normal erythropoeitin, do not mimic the normal differentiation entirely, it is possible that further alteration in the structure of the chromatin are brought about by the initial gene expression when induced by erythropoeitin. Thus there could be a cacade system as envisaged by Britten and Davidson (1969) which is believed to operate in cells responsive to steroid hormones.

Results.

The conditions used for induction of interferon using pretreatment with sodium butyrate were those of Dr. M. Johnston (personal communication) and presented in the methods section. Butyric acid reversibly inhibits the growth of Namalwa cells and stimulates interferon production if the cells are pretreated for 48 hours (M. Johnston personal communication).

To investigate the effect of butyric acid one cannot take a direct approach such as can be done with the action of 5'-BrdUrd in relation to substitution into DNA. Butyric acid is not incorporated into a single subcellular fraction (M. Johnston personal communication). Thus the approach taken was to study the effects of butyric acid on cellular functions relative to its stimulation of interferon, in the hope of finding some temporal correlation.

As a preliminary study the requirement for pretreatment was studied to ascertain the period before which butyric acid has no effect. Fig.17 shows a typical experiment. 48 hours pretreatment was used in all further experiments since the most consistent yields are obtained. The kinetics of interferon production following pretreatment with butyric acid were also studied. Fig.18 shows such a preliminary experiment. There is always more interferon produced by butyric acid treated cells compared to untreated cells at all times at which interferon is detectable in the medium.

To attempt to dissect the kinetics of interferon a study with inhibitors of protein and ribonucleic acid synthesis was undertaken (figs.19 and 20). The use of cycloheximide prevents protein synthesis and can be used to determine whether interferon production is confined to a particular period. All interferon titres were determined after 24 hours incubation with cycloheximide present for various periods, being present either for a continuous period or being reversed by washing out. Pretreatment with cycloheximide (fig.20) shows that interferon is made to a lesser extent the longer cycloheximide is present indicating that interferon that is not translated in a particular period cannot subsequently be translated, thus the inactivation of the messenger RNA which is implied by the kinetics cannot be

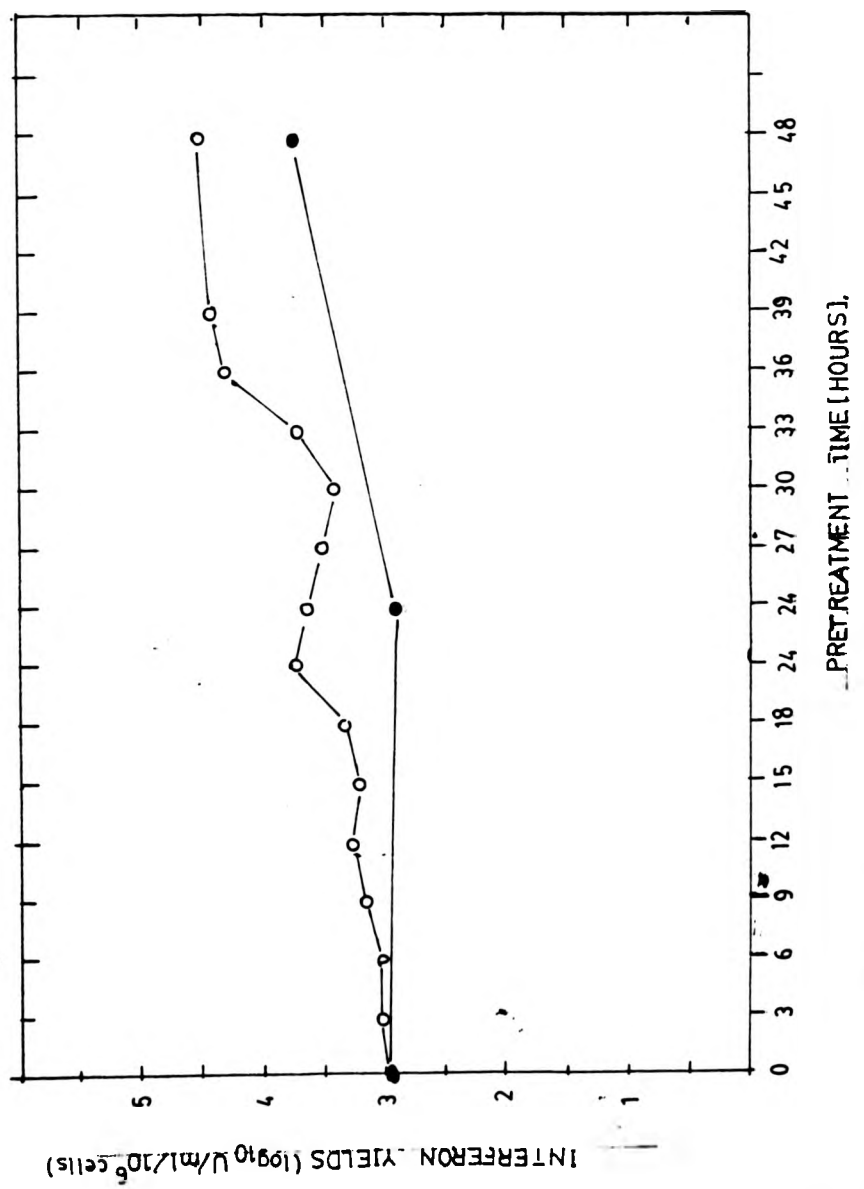
Figure 17.

The stimulation of interferon yields for Namalwa cells.

The stimulation of interferon yields from Namalwa cells by sodium butyrate. Cells treated with 1mM sodium butyrate (○-○), untreated cells (●-●).

lls.

cells


INTERFERON YIELDS (log10 U/ml/10^6 cells)
5
4
3
2
1
0 3 6 9 12 15 18 24 24 27 30 33 36 39 42 45 48
PRETREATMENT TIME (HOURS).

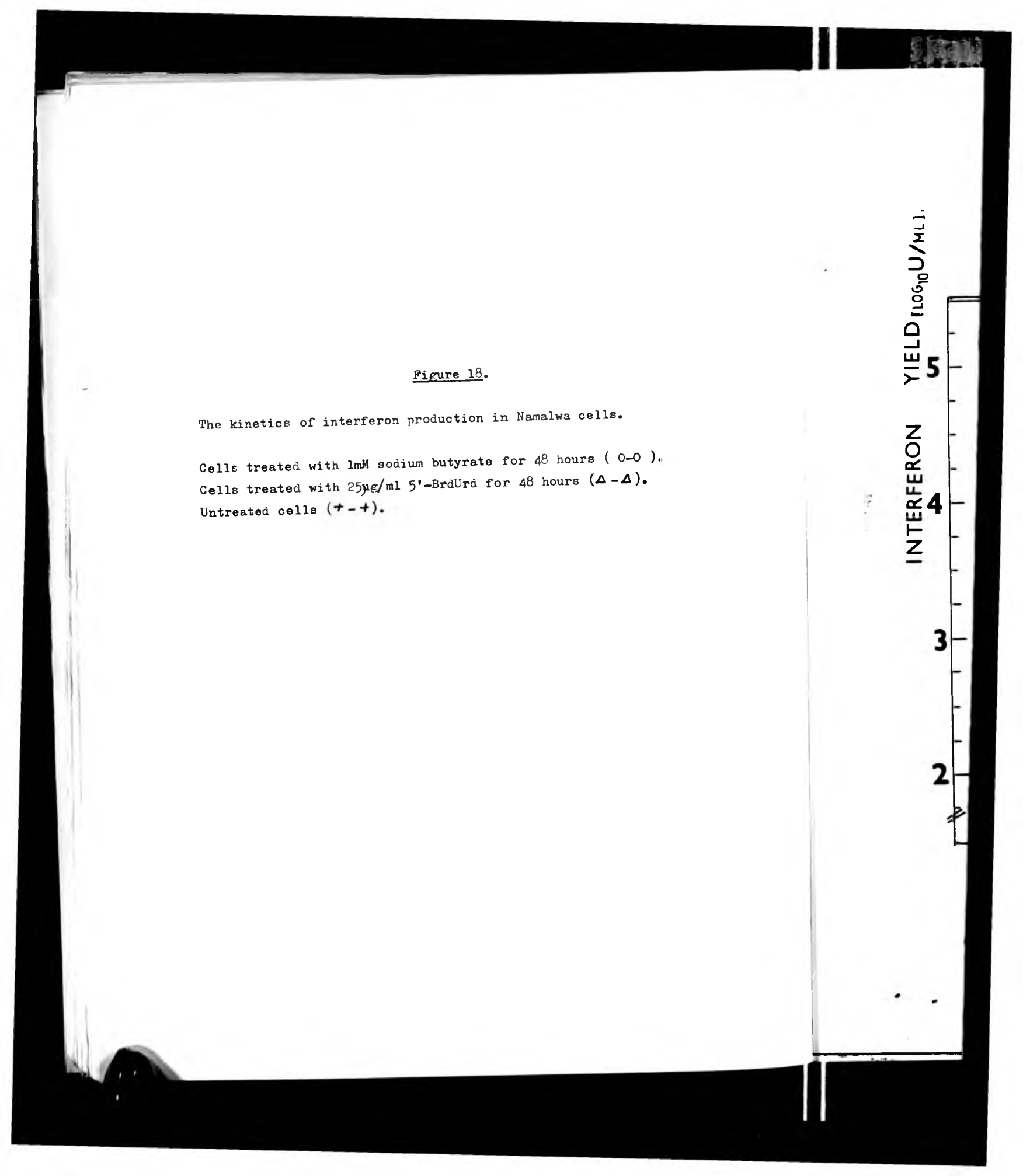


Figure 18.

The kinetics of interferon production in Namalwa cells.

Cells treated with 1mM sodium butyrate for 48 hours (O–O).
Cells treated with 25µg/ml 5'-BrdUrd for 48 hours (Δ -Δ).
Untreated cells (+ - +).

-o).
Δ).

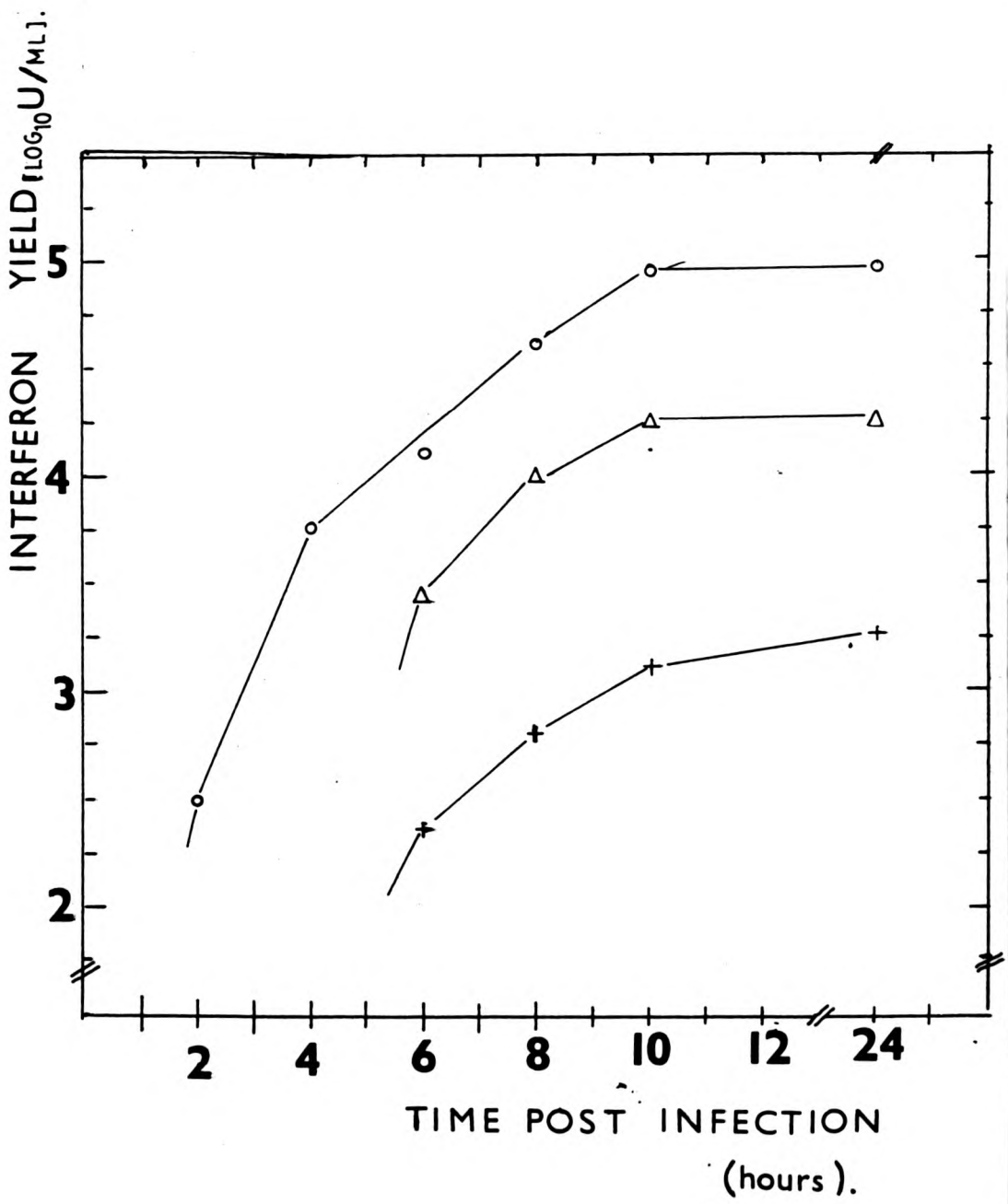

INTERFERON YIELD [LOG10 U/ML].
5
4
3
2
2
4
6
8
10
12
24
TIME POST INFECTION
(hours).

Figure 19.

The effects of actinomycin D and cycloheximide on interferon production from Namalwa cells.

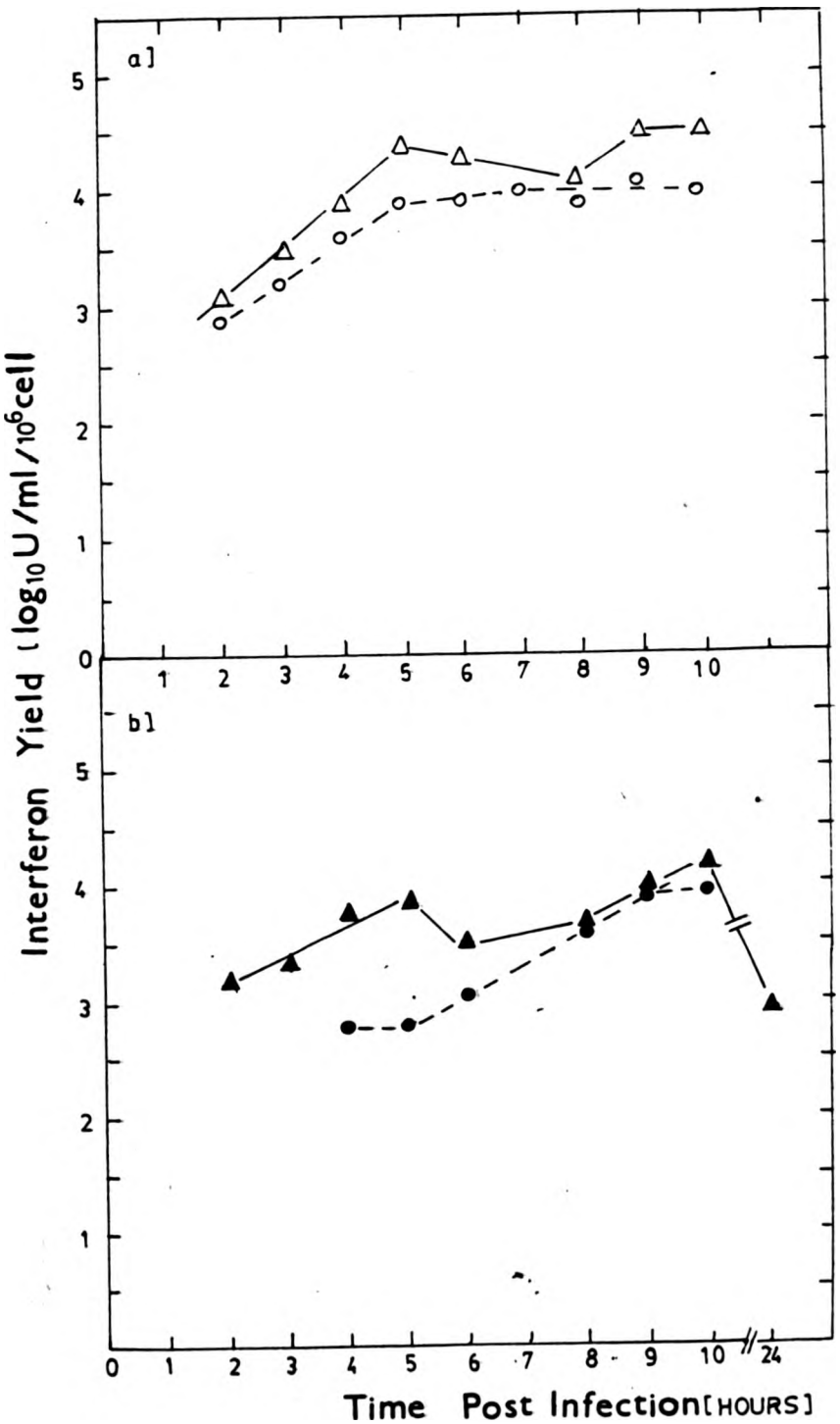
a) Cells treated at one hour intervals post-infection* with actinomycin D (1µg/ml) (Δ-Δ) or untreated (O-O).

b) Cells treated at one hour intervals post-infection with cycloheximide (50µg/ml) (▲-▲) or untreated (●-●).

Interferon yields determined after 24 hours, the inducer being Sendai, 100HA/10^6 cells being added at time zero.

*e.g. 3 hour point with actinomycin D represents the interferon titre at 24 hours with actinomycin present for the period 3-24 hours post-infection.

One of three similar experiments.



with

with

cer

o.

interferon

the period

Figure 20.

The effects of cycloheximide on interferon production from Namalwa cells.

a) Cells treated with cycloheximide (50µg/ml) (Δ -Δ) or untreated (O–O).

All cells were infected with 100HAU/10^6 cells of Sendai virus at time zero. The cycloheximide was washed out (3x with PBS) of samples of a stock culture at each hour post-infection and medium + Sendai replaced.
Interferon yields determined after 24 hours.

b) Cells treated with cycloheximide (Δ -Δ) or untreated (O-O).

Cells were treated with cycloheximide (50µg/ml) for two hour periods at intervals following infection with 100HAU/10^6 cells of Sendai virus. The cycloheximide was then removed by washing (3x with PBS) and medium + Sendai replaced. Untreated cells were mock treated with RPMI 1640 medium + 2% PBS and infected with 100HAU/10^6 cells of Sendai virus, then washed and re-infected as per treated cells.
Interferon yields determined after 24 hours.

One of three similar experiments.

Interferon Yield ($\log_{10}$U/ml/10^6cells)

or

Sendai virus
h PBS) of
and medium +

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for two hour
10^6 cells of
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cells were
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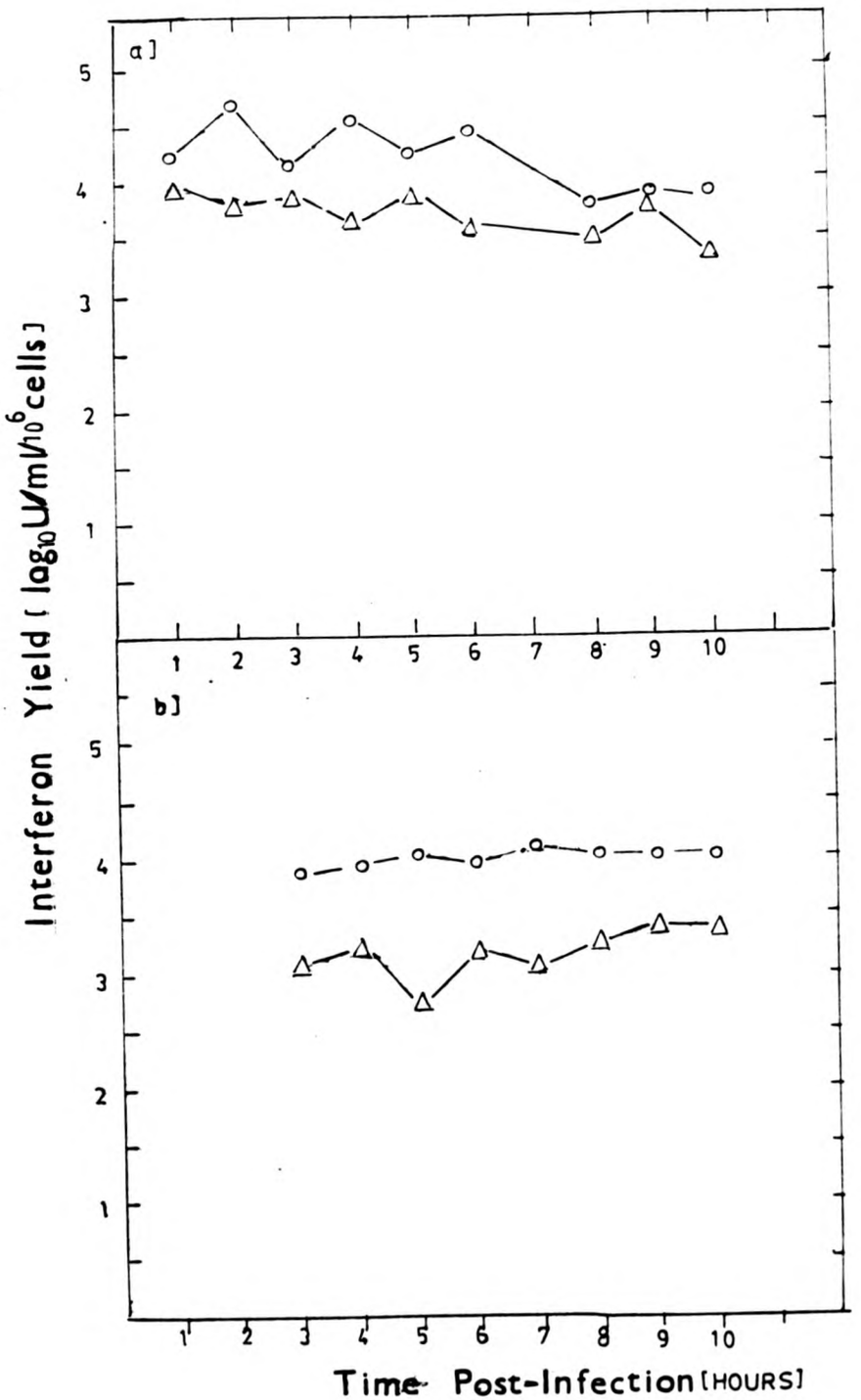
infected as



inhibited and furthermore it does not appear to be altered by treatment with butyric acid as the decline in production is parallel to the untreated case. If treatment is post induction, no change in the interferon produced by 24 hours occurs no matter at what time the cycloheximide is added (fig.20b). The effect of actinomycin D, which inhibits cellular RNA synthesis, cannot be reversed and therefore actinomycin D can only be used post induction. Actinomycin D inhibits interferon production only at early times (fig.19). Since the time courses of release of interferon in the presence of cycloheximide is similar to the release of interferon from untreated cells it seems that interferon is released soon after synthesis.

Thus it seems that the overall rate of interferon production is not altered by butyric acid treatment. Evidence for an increased level of transcription has been presented (Morser et al 1979). The interferon mRNA in butyric acid cells is present at higher levels, as judged by translation of poly A containing RNA extracts of whole cells or polysomes. Further it was shown that interferon synthesis declines after 9-10 hours because the interferon mRNA is found in association with polysomes at its maximal concentration at this time and at lower concentrations before and after this time. The effect of actinomycin D (fig.19) suggests that interferon mRNA is made maximally by about 5 hours since addition of actinomycin D after this time has no effect on the interferon titres obtained.

Since this is clearly at odds with the data derived from measurement of the interferon mRNA levels but in agreement with other data (Zoon et al 1979) it is suggested that actinomycin D either has some secondary effect (apart from direct inhibition of RNA synthesis) or the effect of actinomycin D may be complicated by a processing step required in the formation of active interferon mRNA.

There is no change in the amount of Sendai virus produced after butyrate treatment of cells. Semliki Forest virus grows to similar extents whether the cells are treated with butyrate, 5'-bromodeoxyuridine or are untreated (Table 13). Little change in the effect of Sendai on cellular macromolecular synthesis is seen with butyric acid (fig.21). The figures are quoted in percentages since butyric acid substantially depresses cellular macromolecular synthesis (figs.22 and 23).

Table 13. The effect of sodium butyrate and BrdUrd treatment on SFV growth.

A). TCA precipitable uridine corporation expressed as Infected:Mock infected ratio.

	Hours post infection*							
Treatment	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
None	178	452	1085	432	492	442	349	276
BrdUrd (25 µg/ml)	184	332	778	1011	695	426	409	264
Sodium Butryrate(1mM)	342	620	578	451	446	317	209	151

B). TCA solubles determination

	Hours post infection*							
Treatment	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10
None	62	56	84	68	140	25	63	83
BrdUrd (25 µg/ml)	74	68	74	87	107	86	95	84
Sodium Butyrate(1mM)	68	111	105	85	120	133	102	90

* Namalwa cells infected with 20 pfu/cell of SFV and subsequently labelled with 10 uci/10^7 cells of ^{3}H-uridine for 1 hour.
Actinomycim D (1 µg/ml) was added at t=0.

Means of three experiments with duplicate determinations.

Figure 21.

The effect of sodium butyrate on cellular macromolecular synthesis post-infection.

All cells were infected with 100HAU/10^6 cells of Sendai virus.
Panel A gives the data for the incorporation of ^{3}H-thymidine (250μCi/100ml) .
Panel B gives the data for the incorporation of ^{3}H-uridine (250μCi/100ml).

a) Incorporation into TCA precipitable material

● - ● Butyrate treated cells.
O - O Untreated cells.

b) Incorporation into TCA soluble material

▲ - ▲ Butyrate treated cells.
△ - △ Untreated cells.

Incorporation was determined as the percentage of the incorporation of radiolabel into samples compared with the incorporation into the butyrate treated samples taken at three hours post-infection.
Butyrate treated cells were treated with 1mM sodium butyrate for 48 hours prior to infection.
Means of 3 determinations.

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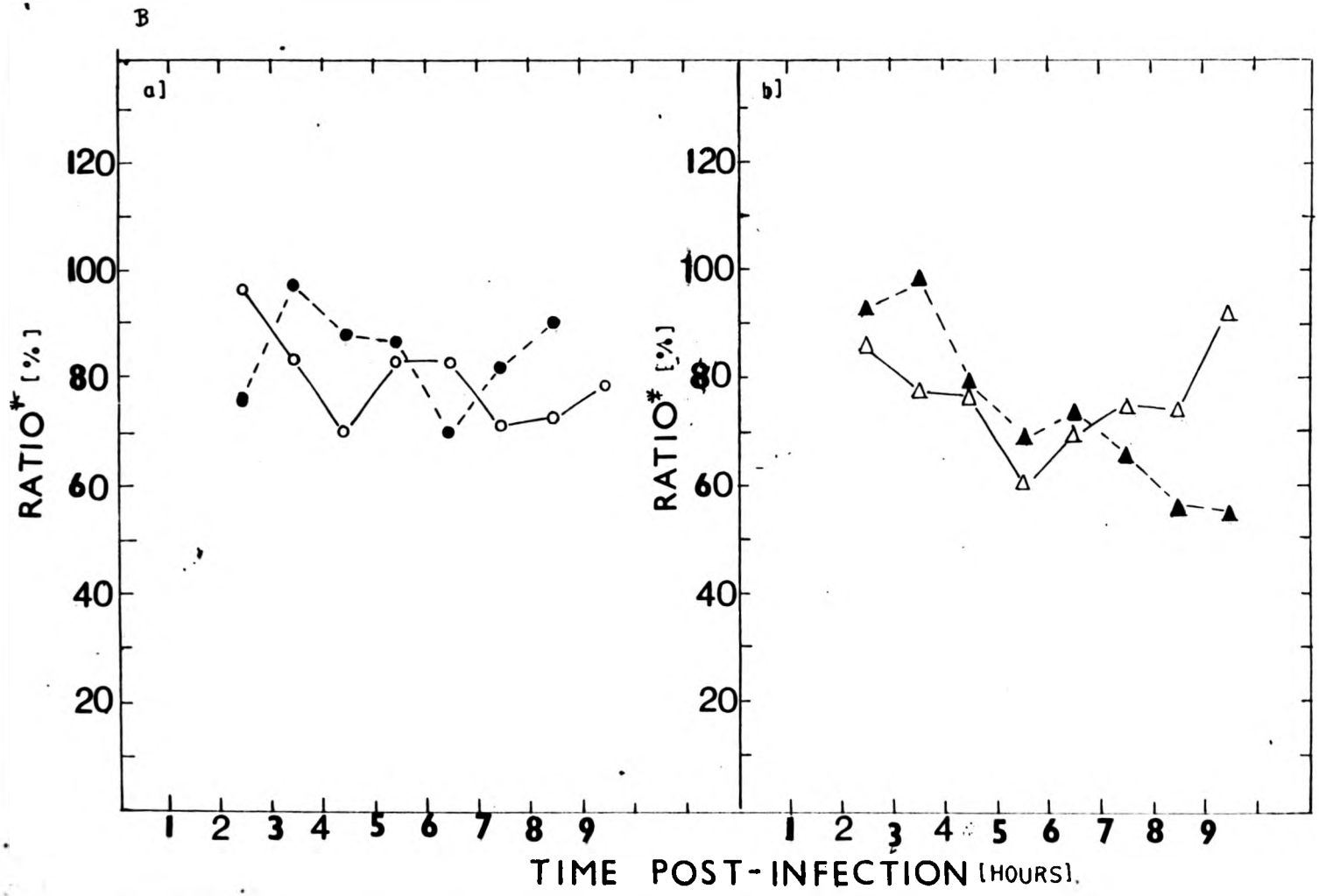

B
a]
b]
120
100
80
60
40
20
RATIO* [%]
1 2 3 4 5 6 7 8 9
TIME POST-INFECTION [HOURS]

Figure 22.

Cellular macromolecular synthesis during pre-treatment of Namalwa cells with sodium butyrate.

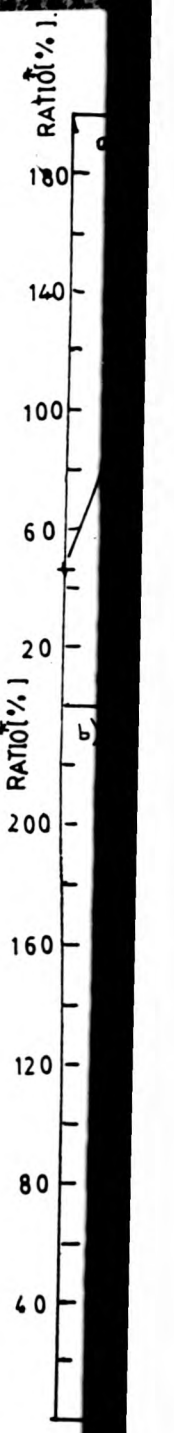
a) Incorporation of ^{3}H-thymidine.

b) ^{3}H-uridine.

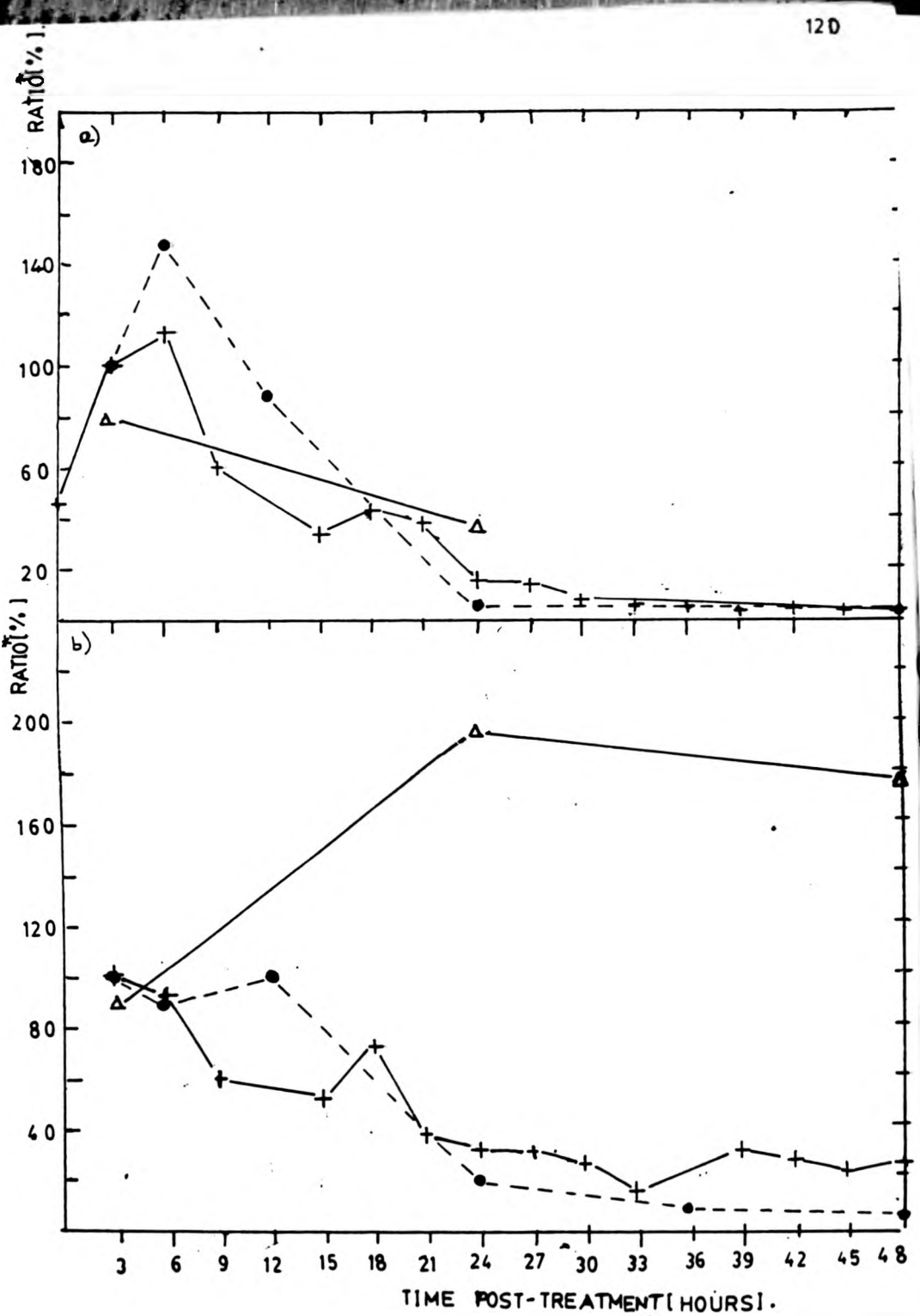
c) ^{35}S methionine.

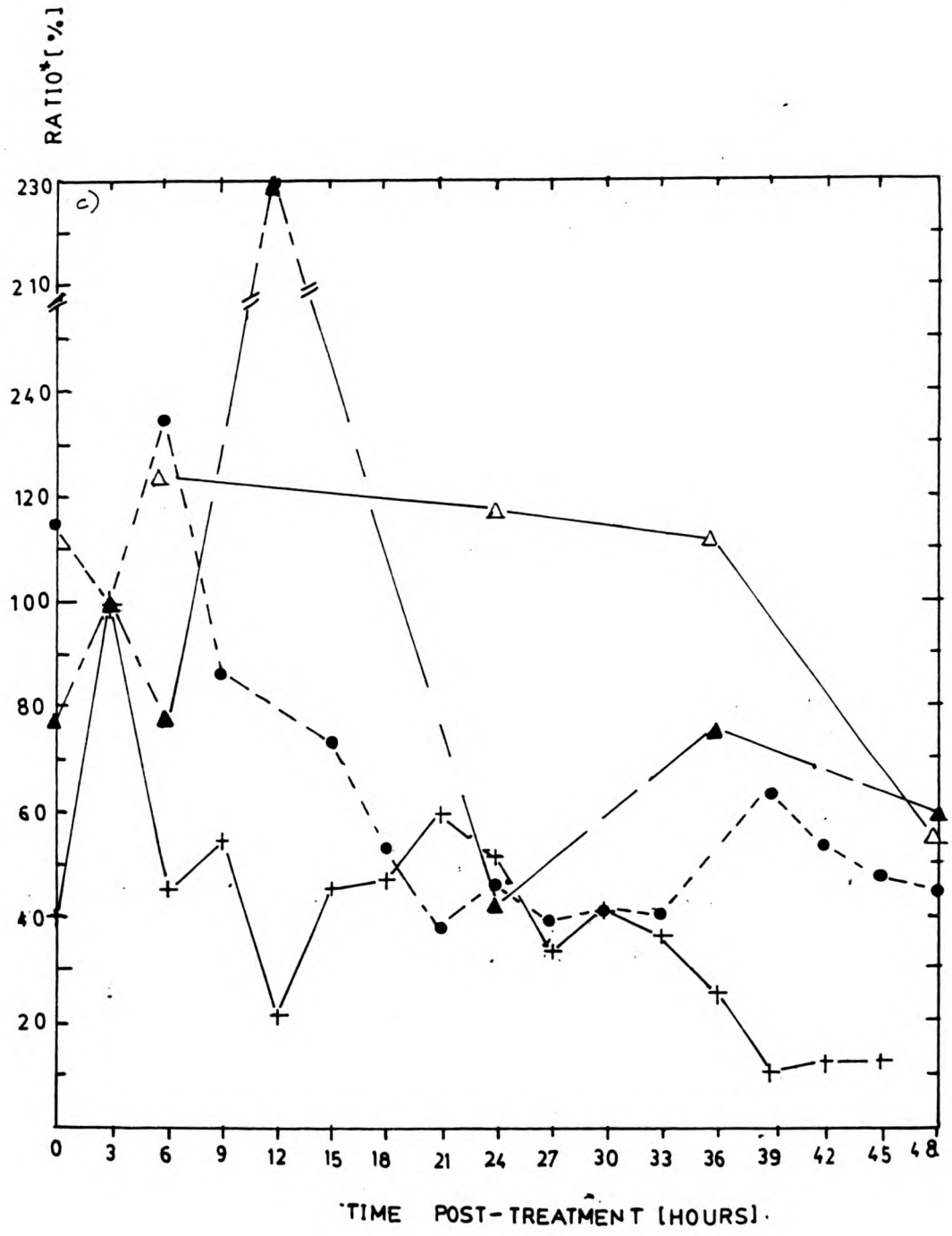
treated cells ▲—▲ / ●—● (2 or 3 determinations)

untreated cells + + / △ △



ations)


RATIO[%]
a)
180
140
100
60
20
RATIO[%]
b)
200
160
120
80
40
3
6
9
12
15
18
21
24
27
30
33
36
39
42
45
48
TIME POST-TREATMENT[HOURS].


RATIO* [%]
c)
230
210
240
120
100
80
60
40
20
0
3
6
9
12
15
18
21
24
27
30
33
36
39
42
45
48
TIME POST-TREATMENT [HOURS]

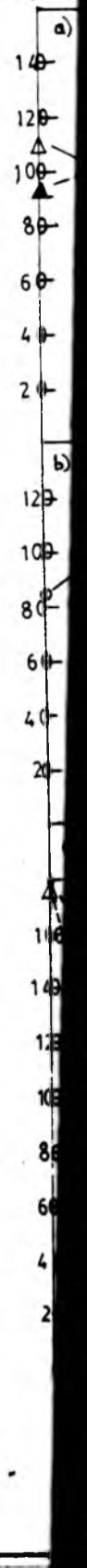


Figure 23.

TCA soluble determinations from radiolabel determinations of cellular macromolecular synthesis.

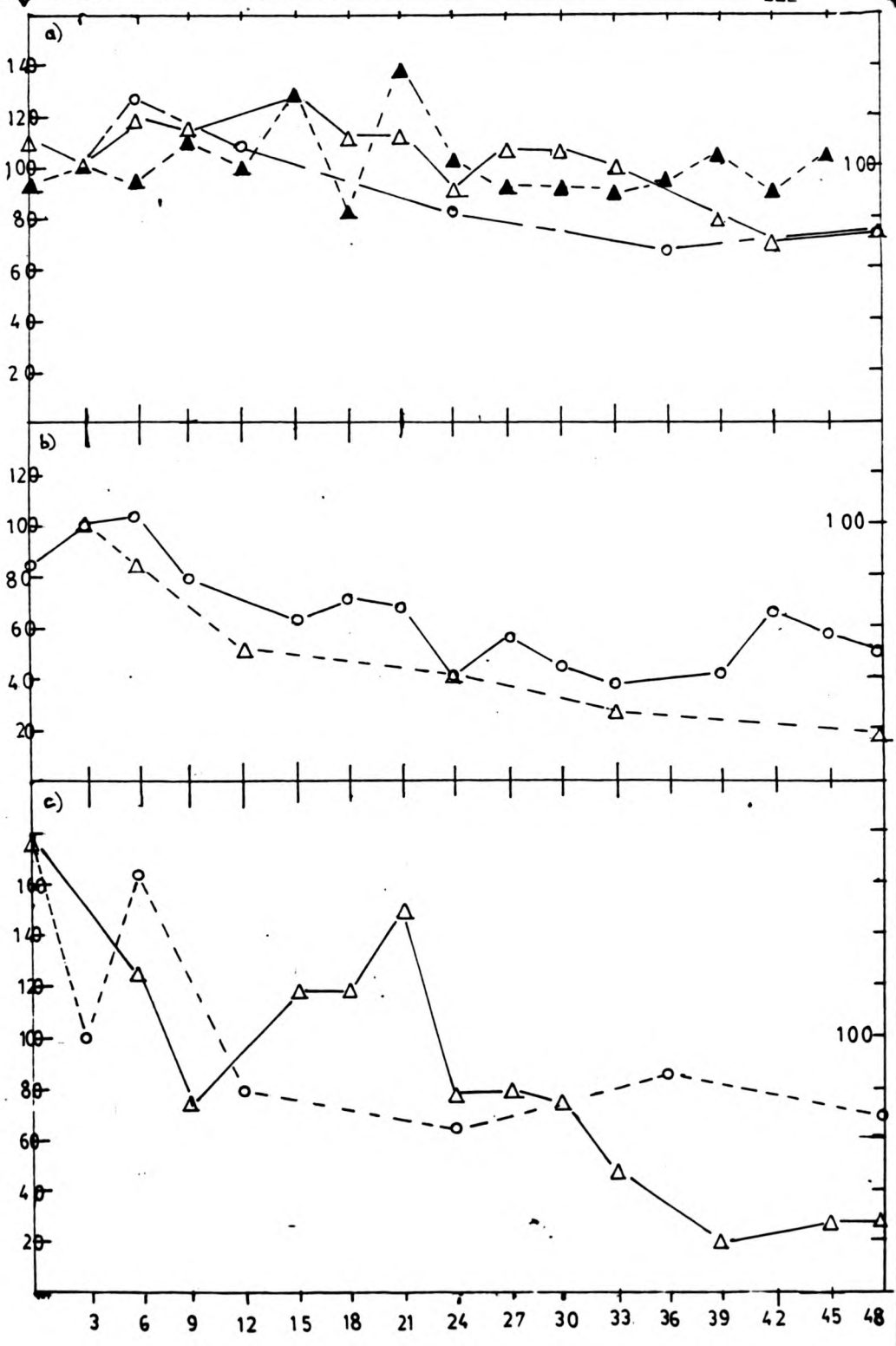
a) thymidine.
b) uridine.
c) methionine.

▲-▲ Namalwa cells treated with 1mM butyrate.

△-△ Untreated cells.

o-o

Mean of two or three determinations.


a)
b)
c)
3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48
TIME POST-TREATMENT [HOURS].

Protein synthesis is least affected being reduced to 60% of normal. DNA synthesis is largely inhibited, being reduced to a few percent of that in untreated cells. Overall it is suggested, as far as can be ascertained, that the viral infection is largely unaltered. There appears to be no change in the proteins made in butyrate treated cells compared to untreated cells. Thus it appears that induction is the same and that the subsequent interferon production is enhanced contrary to the general effect of butyric acid on cellular protein synthesis.

The inhibition of DNA synthesis is not unexpected considering that the cells do not divide in the presence of butyric acid. The residual DNA synthesis could result from DNA repair synthesis by repair enzymes. The DNA per cell was quantitated by microdensitometry to determine whether distribution of DNA contents per cell was similar to untreated cells (fig.24). It is clear from the figures that while untreated cells show a biphasic distribution of DNA contents/cell, this distribution has only one peak for treated cells. The densities measured represent qualitative measurements of the quantity of DNA per cell nucleus. Even so it is clear that the second phase in untreated cells contains about twice the density of stain and therefore twice the quantity of DNA per cell. These cells therefore represent cells in G2 phase. Thus an untreated population contains cells in G1 phase (normal DNA content per cell), cells in G2 phase (twice normal DNA content per cell) plus some cells in S phase (cells containing DNA contents between one and two times normal DNA content, exclusive). Also before division cells can contain twice the DNA content of cells that are in G1 phase. The single peak of DNA contents/cell for the treated cells is indicative of cells in G1 phase. This 'freezing' of the cells in a single phase occurs relatively soon after addition of butyric acid (fig.25). Thus it seems that the growth inhibition of butyric acid is a result of an inhibition of the transition of the cells from G1 to S phase.

The possibility that butyrate treated cells may be smaller than untreated cells, indicated by attempts to determine the protein and DNA contents of cells (Table 14), was investigated further. Using a Coulter counter reproducible reduction in the cellular volume of butyric acid treated cells compared to untreated cells (fig. 26) was shown. Interestingly, some of the cells treated with butyric acid are smaller than the smallest untreated cell. This could

Figure 24.

The DNA content of Namalwa cells.

Determined by measurement of relative (Feulgen) stain of individual cell nuclei.

a) Untreated cells

b) Treated cells (1mM sodium butyrate for 48 hours).

for

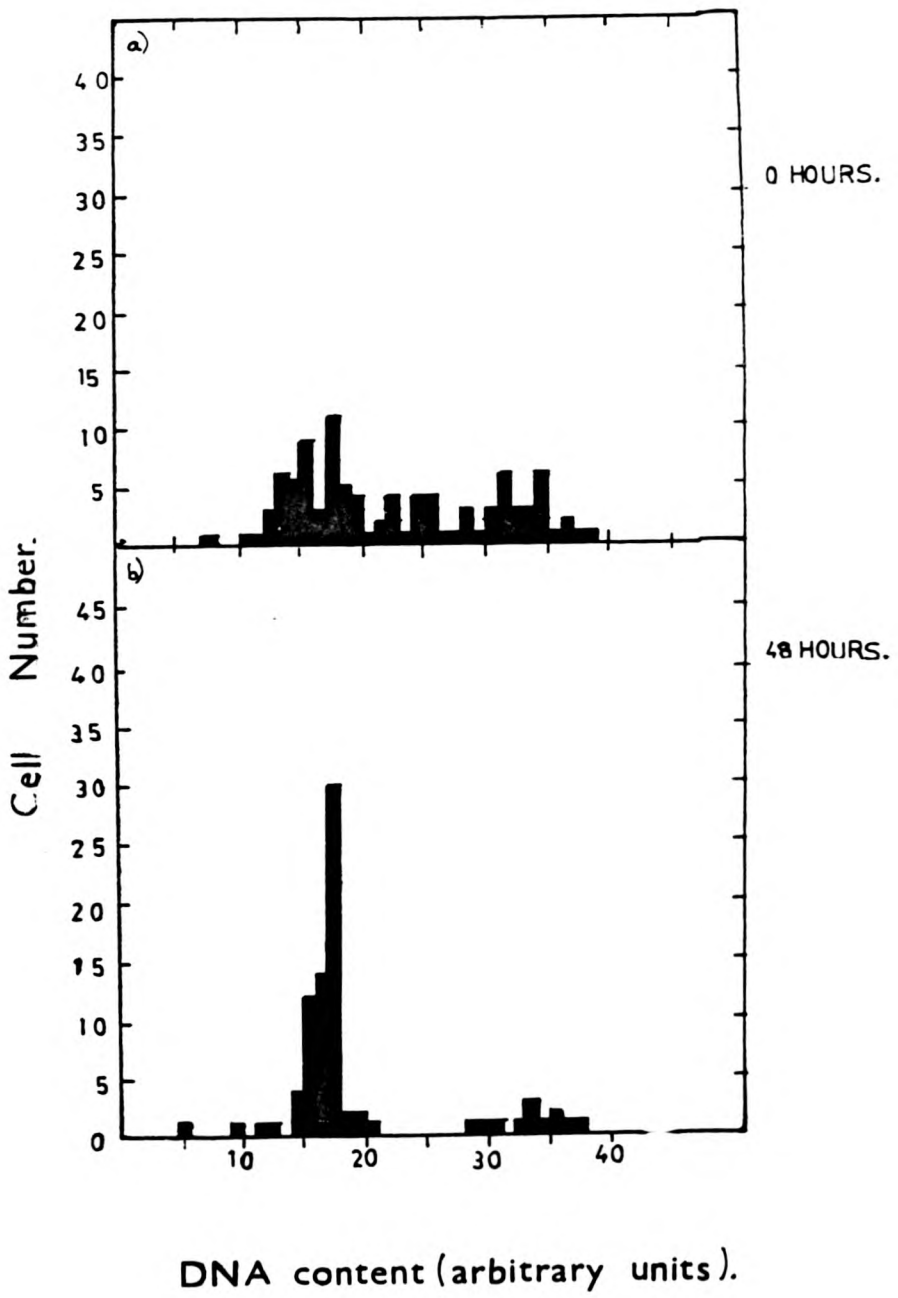
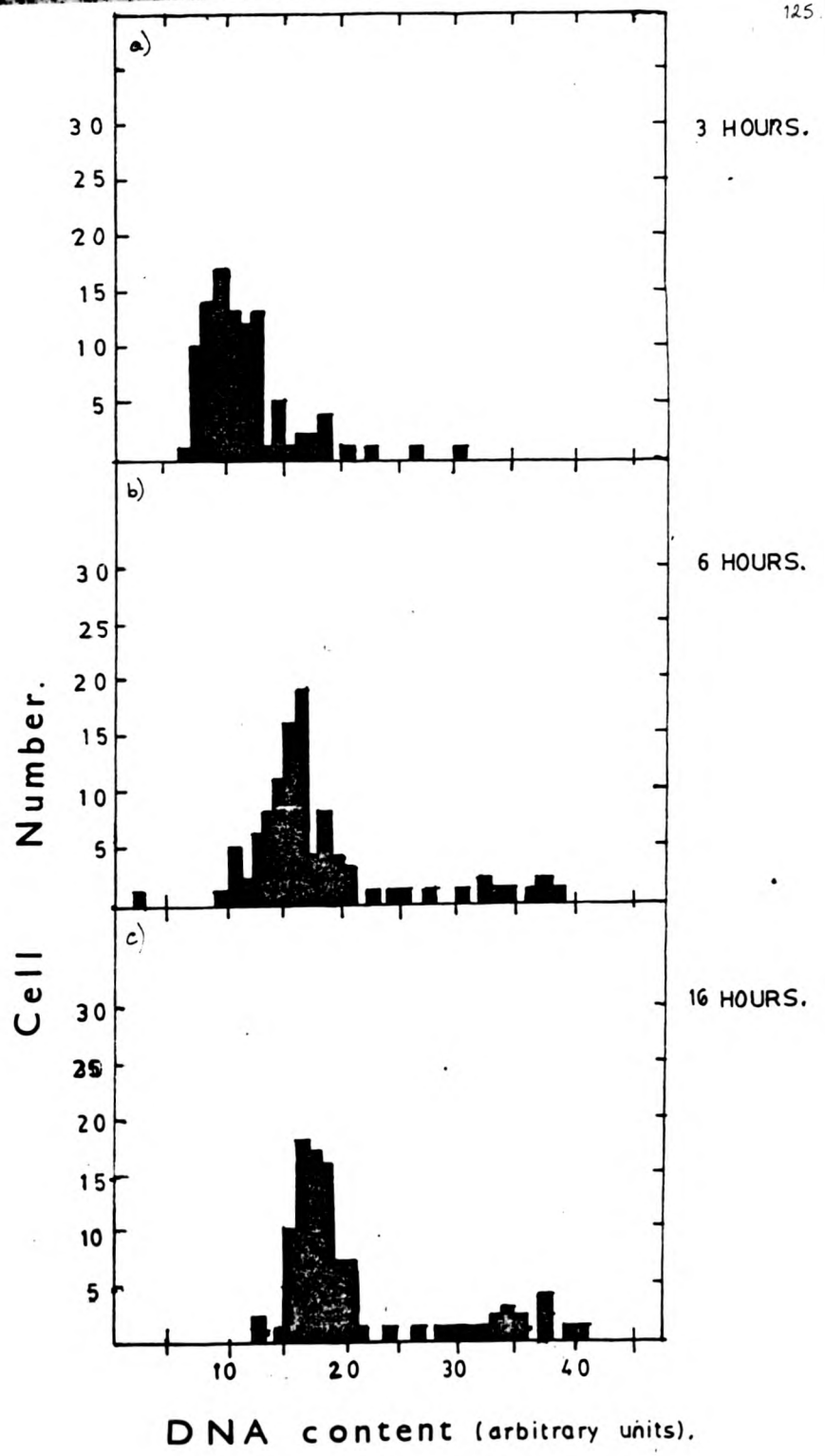


Figure 25.

The DNA content of Namalwa cells.

Determinations of the relative Feulgen stain of individual cell nuclei

a) 3 hours treatment (1mM sodium butyrate).
b) 6 hours treatment (1mM sodium butyrate).
c) 16 hours treatment (1mM sodium butyrate).
d) 24 hours treatment (1mM sodium butyrate).
e) 32 hours treatment (1mM sodium butyrate).


a)
3 HOURS.
b)
6 HOURS.
c)
16 HOURS.
Cell Number.
30
25
20
15
10
5
10
20
30
40
DNA content (arbitrary units).

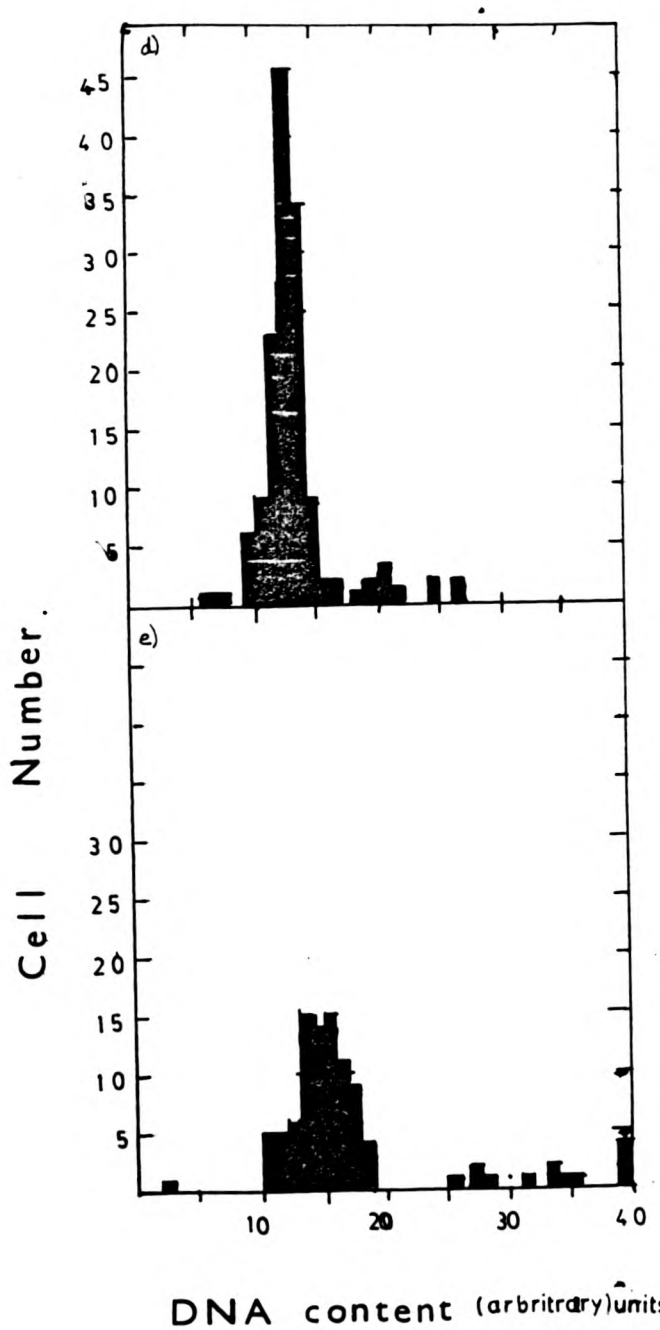

d)
45
40
35
30
25
20
15
10
5
24 HOURS.
e)
30
25
20
15
10
5
32 HOURS.
Cell Number.
10
20
30
40
DNA content (arbritrary)units).

Table 14. Chemical determinations of the protein and DNA content of Namalwa cells.

Protein (pg/cell)		DNA (pg/cell)	
Untreated cells	Butyrate treated cells *	Untreated cells	Butyrate treated cells *
17.3	13.8	5.2	1.5
14.1	13.7	11.5	4.7
37.6	23.0	13.3	3.9
36.0	14.0	14.4	5.9
Mean 26.3	16.2	11.1	4.0

*Cells treated with 1mM sodium butyrate for 48 hours.

Figure 26.

The effect of sodium butyrate on cell size.

a) Namalwa cells treated (Δ-Δ) with 1mM sodium butyrate for 48 hours or untreated (□-□) and infected with 100HAU/10^6 cells of Sendai virus for 2 hours (closed symbols) or uninfected (open symbols).
Cell density was counted in a Coulter counter at A=32 C=1 using a 70um pore. The closed circles represent a 12.92um diameter latex bead standard and the plotted lines are cumulative data from succesive 0.1ml samples of cells diluted 1:4 in Isopon. The channel number is related to the cell size (assuming a spherical shape) - see Methods page.

b) Namalwa cells treated for 15 hours (▲-▲) or 32 hours (●-●) with 1mM sodium butyrate or untreated (■-■).

Peak and mean volume for the cells as derived from the traces in fig a) and b) are shown following fig b).

ate

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32
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he traces

	Peak volume(μ^3)	Median volume(μ^3)	Median diameter* (μ)
a) Untreated and uninfected cells.	740	730±270	11.2±1.2
Butyrate treated (48 hours) and uninfected cells.	410	445±165	9.6±1.0
Untreated and infected (100HAU/10^6 cells) cells.	665	765±300	11.4±1.2
Butyrate treated (48 hours) and infected (100HAU/10^6 cells) cells.	465	475±155	9.8±0.8
b) Butyrate treated (32 hours) and uninfected cells.	615	590±380	10.4±2.1
Butyrate treated (15 hours) and uninfected cells.	472	545±285	10.2±1.5
Untreated control.	760	870±370	11.9±1.5

a) Calculated on a line at 1.8 relative density.
b) Calculated on a line at 4.0 relative density.

* assuming a perfect spherical shape.

Figure 27.

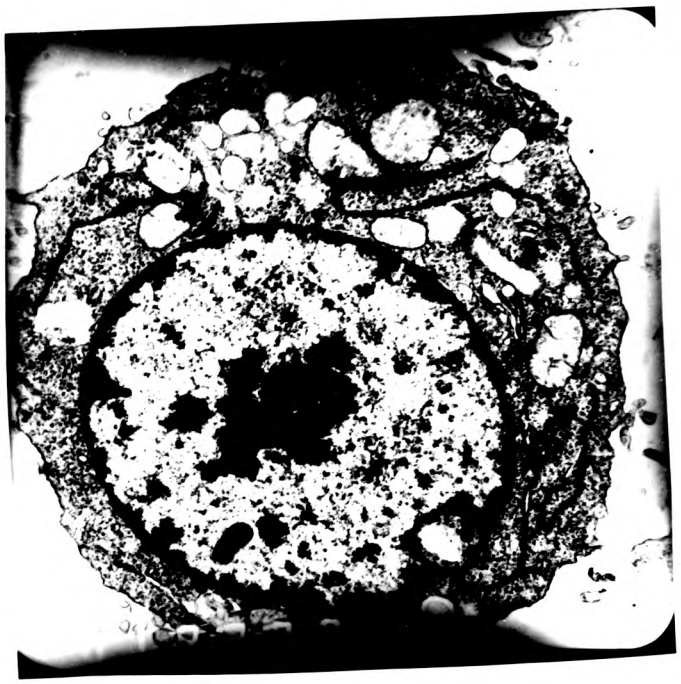
Transmission electron microscopy of Namalwa cells.

a) Namalwa cells treated with sodium butyrate (1mM, 48 hours) Magnification x 11,000.

b) Untreated Namalwa cells. Magnification x 11,000.

c) Particles observed in a single infected cell (x 45,000).

a)

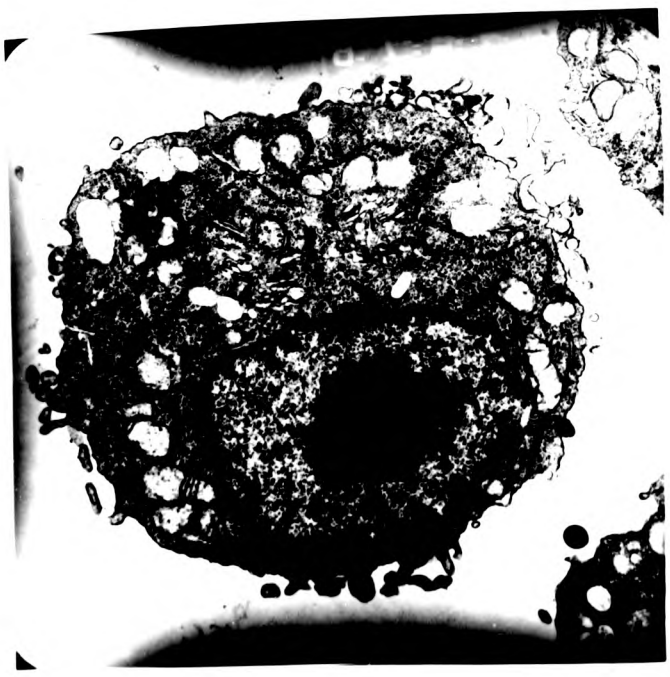


mM, 48 hours)

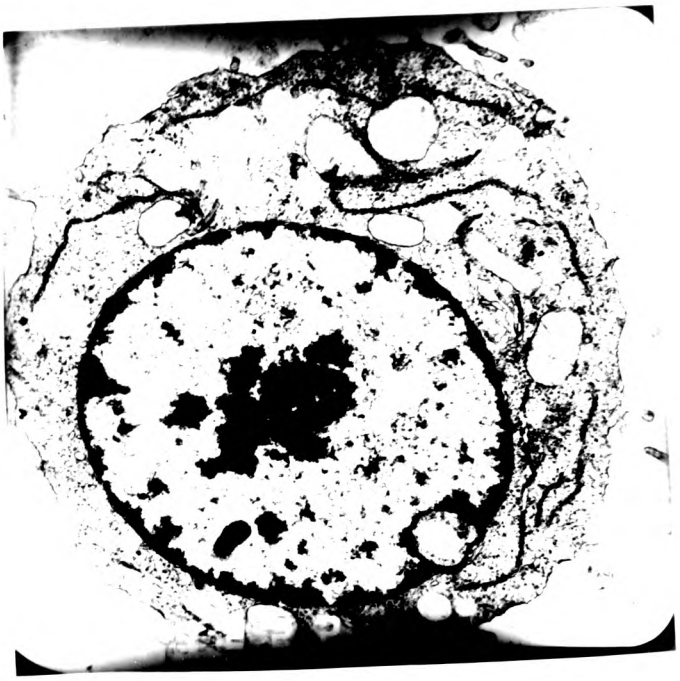
000.

(x 45,000).

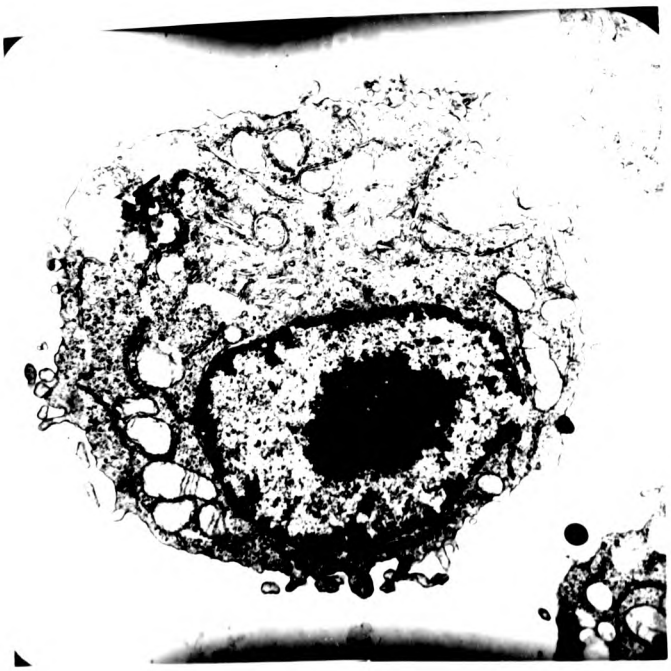
b)

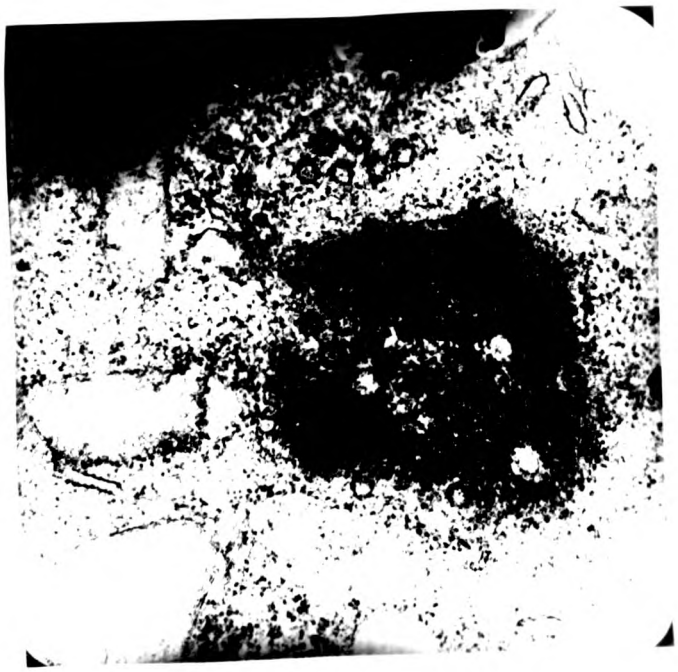


a)

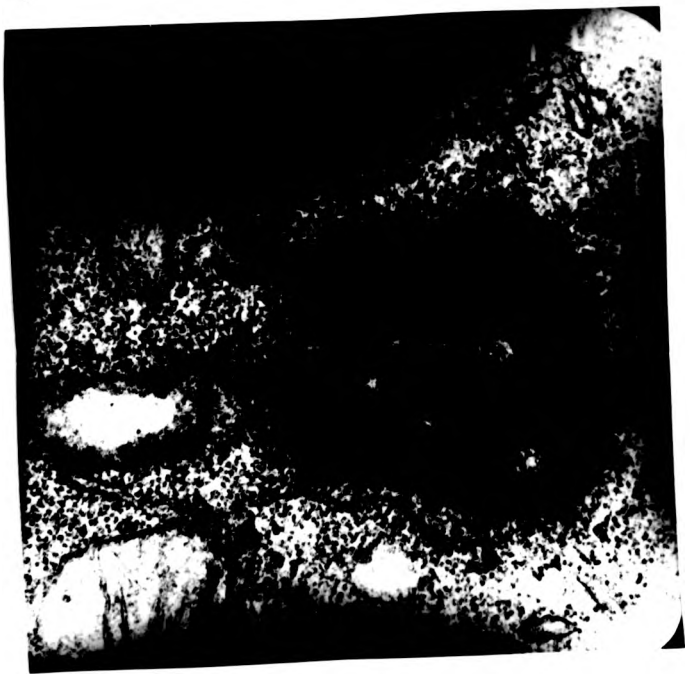


b)





c)



reflect a change in morphology i.e. a more filamentous state which would be recorded as a smaller volume by the Coulter counter whilst retaining the same surface area. Transmission electron microscopy of thin sections did not reveal any changes in cellular morphology and no striking changes in intracellular components (fig.27). No viral particles were present in the sections of infected cells studied with the possible exception of a single cell (fig.27) (out of a few hundred examined). These particles are about 200nm in diameter but whether they represent Sendai virions or Epstein-Barr viral particles or other particles was not determined further. The lack of particles in the majority of cells suggest that if they are not artifactual, then particles of such low frequency would be hard to study. Scanning electron microscopy revealed no dramatic differences between treated and untreated cells in their surface morphology (fig.28). However two populations of cells seem to be present regardless of treatment. One type of cell has a smooth cell membrane (fig.28a) and the other appears 'ruffled' (fig.28b). The basis for this morphological difference is not known. Clearly, though, butyric acid treatment does not significantly affect cell morphology: the cells are merely smaller. The fact that they are smaller than the smallest untreated cells support that cellular membrane properties could be altered, resulting in a changed distribution of the plasma membrane. An indirect study of the plasma membrane was undertaken by testing the permeability of the cells to rubidium-86. This ion is taken up in the same manner as potassium. Both the rates of influx of this ion and the rates of efflux, after a period of 1.5 hours prelabelling, were measured at times between 2 and 60 minutes after addition or removal of extracellular rubidium (Table 15). The results show that uptake of rubidium is decreased after treatment with butyric acid but that the efflux rate is unaffected.

Discussion.

The results show that sodium butyrate acts in a similar fashion to 5'-bromodeoxyuridine as far as the effect on the kinetics of interferon production, inhibiting cellular growth and having no effect on the growth of both the inducing virus and a permissive virus, SFV.

Figure 28.

Scanning electron microscopy of Namalwa cells.

a) and b) represent a sample of the two differing morphological types of Namalwa cell. Magnification x 200,000 approx.

c) and d) as above with cells treated with 1mM sodium butyrate for 48 hours.

The photographs presented here were made by Dr. J. Beesley (Dept. of Electron Microscopy, Wellcome Research Foundation Laboratories) using samples of Namalwa cells prepared by myself.

ng morphological
x 200,000 approx.

sodium butyrate

J. Beesley (Dept. of
n Laboratories) using

a)

g morphological
200,000 approx.

odium butyrate

Beesley (Dept. of
Laboratories) using

a)

б)

b)

c)

c)

d)

d)

Table 15. The effect of pretreatment of Namalwa cells with sodium butyrate on the permeability to rubidium.

86-Rubidium (1miCi/ml, 180ng ^{86}Rb/ml on 23/7/79).

			Rate
Influx	Treated cells	417cpm^2	3.0×10^{-15} moles/min
	Untreated cells	766cpm^2	7.2×10^{-15} moles/min
			$t_{\frac{1}{2}}$**
Efflux*	Treated cells	$5.3 min^{-1}$	46 mins
	Untreated cells	$6.2 min^{-1}$	50 mins

The above was the average of 3 experiments and corrected for decay ($t_{\frac{1}{2}}$ = 18.66d), using 1.00×10^6 cells/ml.

* Efflux is determined as the $\log_{10}$ rate since the kinetics were exponential.

** $t_{\frac{1}{2}}$ is the time for the intracellular rubidium to decline to half of the first determination, obtained by extrapolation.

However the similarities end here since sodium butyrate has no obvious site of action. Cells are arrested in G1 phase whereas cells treated with 5'-bromodeoxyuridine still grow, albeit slowly. It is possible that cells only make interferon in G1 phase and thus virtually all the cells in a butyrate treated culture do, whereas only a proportion make interferon in an untreated culture. This seems unlikely considering the increase in interferon yields is generally about 10 fold, so the G1 phase would have to last only about 10% of the cell cycle for this mechanism to be the sole basis for the increased yields. It has been shown elsewhere (Morser *et al* 1979) that the interferon mRNA levels detectable by oocyte assay are increased in butyrate treated cells. However the increase in quantity of the mRNA only accounts in part for the increase in interferon activity detected.

The mechanism of inhibition of DNA synthesis is not obvious but may result from changes in the binding of chromosomal proteins in the presence of this lipophilic agent, be it direct or indirect. This has been proposed for other systems where DNA synthesis can also be inhibited *in vitro* (Hagopian *et al* 1977). Acetylation of the histones may be involved (see introduction) and certainly histones are acetylated in the presence of butyric acid in Namalwa cells (J. Morser personal communication). Since many of the functions of chromosomal proteins remains unknown, this is a difficult area to study. However it is clear that these proteins play a role in the regulation of gene expression, so an alteration in these proteins offers a potential mechanism for changes in the expression of the interferon gene.

Other general mechanisms have been proposed for the action of butyric acid. Since it alters the properties of cell membranes, such an activity could result in alterations in cellular metabolism. Butyric acid appears to alter membrane permeability in Namalwa cells but does not alter cellular morphology. Whether this altered permeability is significant as regards cellular gene expression remains to be demonstrated. It is perfectly possible that this effect is unrelated to the effect on interferon expression or that it results from the inhibition of DNA synthesis.

Work with *in vitro* systems derived from Namalwa cells could be an important step in elucidating the role of butyric acid directly on general cell expression.

CONCLUSION.

The work above shows that at the present time the only method of obtaining consistently high yields of interferon lies with treating Namalwa cells with suitable chemical treatments. The basis of the alteration in cellular macromolecular synthesis has not been uncovered. Since the effects of 5'-bromodeoxyuridine and sodium butyrate on all but interferon production are widely disparate it seems unlikely that alteration in a single mechanism in interferon production is indicated. Indeed it has not been ruled out that both chemicals act without altering rates of transcription or indeed that they act in more than one way to promote interferon production. The apparent complexity of the interferon system itself would indicate that several alterations in cellular functions might promote interferon production.

The effect on interferon production can be added to the many and varied activities of these two biologically active compounds. Several more trivial explanations of their activity have been ruled out - they do not alter the activity of the inducer, the stimulation of interferon synthesis is not a result of a general stimulation of protein synthesis or an increase in cell size. There does not appear to be any subset of cells which are major interferon producers, thus it is unlikely that the treatments alter the relative proliferation of such putative subsets. The time of treatment is also critical - in the case of 5'-BrdUrd presumably the time to be substantially incorporated into cellular DNA, but for butyric acid it is not known why 48 hours is the best time unless it is purely a synchronisation phenomenon. It is likely that during this time fundamental changes in cellular metabolism occur, causing or resulting from changes in the structure and expression of cellular DNA.

An extension of this work into measuring the levels of transcription of the interferon gene is indicated. This would lead to two types of study. Firstly, the purification of interferon messenger RNA might be possible leading to studies on the regulation of the

interferon system and possibly allowing genetic manipulation. Secondly, the action of the chemicals I have used at the level of the genetic material to selectively alter a single protein (and perhaps a single gene) might lead to information on the way they act to alter differentiation and thus on the mechanisms involved in differentiation itself.

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